Fatty acid composition and glycerolipid metabolism during temperature adaptation in the marine cyanobacterium Aphanizomenon sp.

Gashlan, Hana Mohamed A

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Hana Mohamed A. Gashlan

B.Sc. (Jeddah), M.Sc. (U.K.)

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Supervisors

School of Biological Science,

University of Wales Swansea

External: Dr. Terence J. Walton

Internal: Dr. Khalid Abulnaja

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Summary

The nature and mechanisms of adaptive changes in the fatty acid composition of membrane glycerolipids in laboratory cultures of the marine cyanobacterium *Aphanizomenon* sp. induced by temperature transitions similar to those experienced by natural population of *Aphanizomenon* in the Baltic Sea were investigated. At 28° palmitic acid (16:0) predominated (35%) over α-linolenic acid (α-18:3; 23%), whilst in cells grown at 15°, α-18:3 (38%) predominated over 16:0 (25%). Changes in fatty acid composition of the major glycerolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SL) and phosphatidylglycerol (PG) were studied after temperature shifts from 28° to 15° and from 15° to 28°. After lowering the temperature, the level of 16:0 and 18:1 decreased in MGDG, DGDG and SL fractions, whilst the content of 16:3 and α-18:3 increased. In PG however the level of 16:1 and 18:1 increased whilst the level of α-18:3 decreased. After a temperature shift from 15° to 28° the level of 16:0 remained unchanged in MGDG and SL whilst α-18:3 decreased and 16:1 increased. Determination of the positional distribution of fatty acids present in the individual glycerolipid classes using *Rhizopus* lipase hydrolysis established the predominance of C-18 fatty acids at the *sn*-1 position and C-16 fatty acids at the *sn*-2 position; during the adaptation to lower temperature the most prominent change at the *sn*-1 position was an increase in 18:1, 18:3 and 16:3 levels in MGDG. Glycerolipid biosynthesis and metabolism in cultures grown at 28° and during a downward temperature shift were investigated in single and dual radioisotope labelling studies using ^{14}C-acetate and ^{3}H-glycerol. In cultures pre-labelled from ^{14}C-acetate, the pattern of glycerolipid radioactivity following a temperature downshift was

consistent with direct desaturation of C-18 monoenoic fatty acyl chains bound in 18:1/16:1-MGDG species to yield 18:3/16:1-MGDG. Supporting evidence for the operation of this direct desaturation pathway was also provided by a study of the ^{3}H:^{14}C dpm ratios of MGDG species during temperature adaptation after pre-labelling the glycerolipids from ^{3}H-glycerol and ^{14}C-acetate.

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ABBREVIATIONS

$CH_3CO.SCoA$	Acetyl-CoA
ACCASE	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
α	Alpha
NH_3	Ammonia
$AgNO_3$-TLC	Argentimetric TLC
β	Beta
β-KASI	Type I β- ketoacyl ACP synthetase
β-KAS II	Type II β-ketoacyl ACP synthetase
β-KAS III	Type III β-ketoacyl ACP synthetase
HCO_3	bicarbonate
C-1	Carbon number one
C-2	Carbon number two
cm	Centimeter
CoA	Co-enzyme A
Conc.	Concentration
c.p.m	Counts per minute
°	Degree centrigrade
DNA	Deoxyribonucleic acid

DAG	Diacylglycerol
DAG	Diacylglycerol
DGDG	Digalactosyldiacylglycerol
d.p.m	Disintegration per minute
E-biotin	Enzyme bound biotin
C-16	Fatty acid chain with 16 carbon atoms
C-18	Fatty acid chain with 18 carbon atoms
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
Fig	Figure
GLC	Gas-liquid chromatography
g	Gram
H	Histidine cluster
h	Hour
H_2	Hydrogen
IUPAC-IUB	International Union of Pure and applied Chemistry-International Union of Biochemistry
$HOOC.CH_2COSCoA$	Malonyl-CoA
m	Meter
µg	Microgram
µl	Microliter
Mmol	Micromole
v/v	Volume per volume
%	Percentage
µ	Micron

mg	Milligram
ml	Milliliter
min	Minute
MGDG	Monogalactosyldiacylglycerol
GlcDG	Monoglucosyldiacylglycerol
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
N_2	Nitrogen
OFN	Oxygen free nitrogen
Pi	Phosphate
PG	Phosphatidylglycerol
PSI	Photosystem I
PSII	Photosystem II
PUFAs	Polyunsaturated Fatty Acids
K^+	Potassium
PSU	Practical Salinity Units
NADP	Reduced nicotinamide adenine dinucleotide phosphate
Rf	Retention index
rpm	Revolution per minute
RNA	Ribonucleic acid
sn	Stereospecific numbering system
SL	Sulfoquinovosyldiacylglycerol
SQDG	Sulfoquinovosyldiacylglycerol

Tab.	Table
TLC	Thin-layer chromatography
TL	Total lipid
TCA	Tricarboxylic acid cycle
TGDG	Trigalactosyldiacylglycerol
U.V.	Ultra violet
UDP	Uridine diphosphate
λmax	Wavelength of maximum light absorption

CHAPTER ONE

INTRODUCTION

1.1 Morphology and Classification of Cyanobacteria

The cyanobacteria , Cyanophyceae or blue–green algae are the prokaryotic algae. They are more closely related to the prokaryotic bacteria than to the eukaryotic algae, a relationship that led to the drive for their classification as cyanobacteria instead of blue-green algae (Stanier *et al.*, 1977). They are minute organisms, although their mass occurrence in aggregates and filaments makes them clearly visible to the naked eye. There are many examples of their presence in nature as floating filaments and spherical colonies of cynobacteria, which are familiar components of the microscopic community of freshwater and marine phytoplankton. They may occasionally occur in such abundance as to cause a striking green colouration to natural water or to form floating 'mats' at the surface. Such 'water blooms' are frequent during the summer and may persist in tropical lakes and on the surface of calm seas. Cyanobacteria are also widely located on damp rocks, in salt marshes, on the beds of the rivers, on the trunk of trees, and on stones. They form gelatinous masses, furry cushions, skin-like sheets or powdery coverings on the substratum. The colour of these forms, may vary from blackish-green to olive green, orange-yellow or reddish brown, besides the typical blue-green (Fay, 1983).

Cyanobacteria are classified as Gram-negative bacteria and their cell envelope is composed of the outer lipoprotein membrane. This is separated from the inner membrane (plasma or cytoplasmic membrane) by a peptidoglycan layer, which constitutes up to 50% of the dry weight of the cell (Frank *et al.*, 1962; Hocht *et al.*, 1965) In addition, their cells have an intracellular photosynthetic membrane, known as the thylakoid membrane, which contains chlorophyll-a, β-carotene and phycobilisomes, and which incorporates the components of the electron transport

chain including cytochromes, plastocyanin and ferredoxin (Fay, 1983). The plasma membrane and outer membrane contain xanthophylls, but little or no chlorophyll-a or β-carotene (Murata *et al.,* 1981, Omata and Murata 1983, 1984a, Resch and Gibson 1983, Jurgens and Weckesser 1985). Outside the cell wall of most cyanobacteria is a layer of extra-cellular mucilage called the slime layer, sheath or capsule, which protects the cells from drying out and is involved in gliding (Stein, 1973). In some planktonic species, the sheath is of a watery consistency and thus difficult to see. The protoplast shows elementary internal structure (Fig 1.1), the central protoplasm containing circular fibrils of DNA which lack basic proteins (histones). Also found in the central part of the cells are bodies associated with the DNA and postulated to contain RNA (Stewart and Codd,1957). In addition, polyphosphate bodies are present in the protoplasm and contain stored phosphate (Tischer, 1957). In the space between the thylakoids, polyglycan granules are common and contain polysaccharides that are characteristically composed of 14 to 16 glucose molecules (Hough *et al* ., 1952; Frederick,1951). In addition, there is a gas vacuole which is considered as one of the most important parts of the internal cell structure.

The gas vacuole is composed of gas vesicles, or hollow cylindrical tubes with conical ends (Walsby,1972 ; 1973). It does not have a true protein-lipid membrane, being composed exclusively of protein ribs or spirals arranged similarly to the hoops on a barrel. The gas inside it is at a pressure of 1 atmosphere, and it is possible to collapse the gas vesicles by applying pressure to the cells. The membrane of the gas vesicle is quite rigid although permeable to gases, which allows the contained gas to equilibrate with gases in the surrounding solution, but it is able to exclude water (Walsby, 1973) Gas vacuoles may function in light shielding

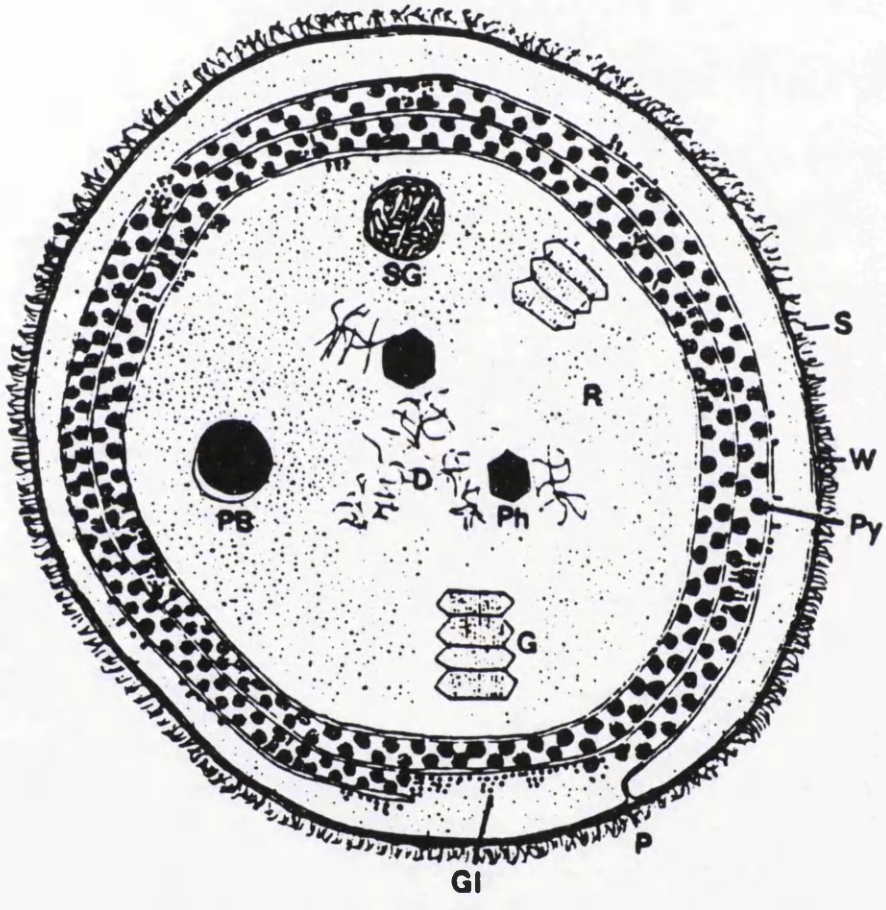


Fig 1.1 : Structural features of a cyanobacterial cell. (D) 'DNA fibrils: (G) gas vesicles;(GI) glycogen granules; (P) plasmalemma; (PB) polyphosphate body; (Ph) polyhedral body; (Py) phycobilisomes; (R) ribosomes; (S) sheath; (SG) structured granules (cyanophycin granule); (W) wall. (Simon,1973).

(Lemmermann, 1910), and species in which the gas vacuoles are found are normally near the surface of the water and therefore exposed to high light intensities.

Gas vacuoles have been shown to have a key role in buoyancy. Cyanobacteria possessing gas vacuoles can be divided into two physiological-ecological groups (Walsby,1972; 1973). The first group develop gas vacuoles only at certain stages of their life cycle, or only in certain types of cells. For example, in *Gloestrichia ghosei* and certain species of *Tolypothrix* gas vacuoles only appear in hormogonia. The hormogonia float when they are released, and it is possible that the buoyancy provided is of significance in dispersal of these stages. The second group comprises planktonic cyanobacteria such as *Aphanizomenon flos-aquae*, and these derive positive buoyancy from their gas vacuoles and float near the water surface and form blooms where they experience optimum light conditions (Walsby *et al.,* 1995).

Cyanobacteria not only vary in their habitats, but also in the range of vegetative structure developed. The principal types of vegetative structure are the unicellular, colonial filamentous and other colonial forms.

The thallus in unicellular species is a single cell, which may be spherical or oval. The largest group among the unicellular genera are the *Chroococcus*. Groups of unicellular cells embedded in polysaccharide matrices and held together in palmelloid colonies may occur. The organisms in this order have a similar morphology, and fatty acid composition (Stanier *et al.,* 1977). The cells of the colony are similar and undifferentiated from each other. In most cyanobacteria, the cells after division remain attached by their walls or are attached together to form colonies. The colonies may either be cubical, spherical, square or irregular depending upon the planes and direction in which the cell divided. In filamentous cyanobacterial species the filament is composed of a chain of cells called the

trichome and is enveloped by a mucilaginous sheath (Evans *et al.*, 1976). These filamentous forms may be classified into two series: those which produce heterocysts such as *A. cylindrica* (Walsby and Nichols, 1969) and those which lack them such as *Trichodesmium* (Carpenter and Capone). Cyanobacteria have been classified by Geitler (1932), Smith (1950) and Drouet (1981) into three orders, Chroococcales, Chamaesiphonales and Oscillatoriales, while other authors, (Fritsch, 1945; Desikachary, 1959 and Bourrelly,1970) grouped cyanobacteria into five orders, Chroococcales, Chamaesiphonales, Pleurocapsales, Nostocales and Stigonematales.

1.2 Characteristic Cyanobacterial Physiology and Biochemistry

Cyanobacteria are of considerable importance in the natural environment as initial colonizers of arid land and as primary producers of organic matter. Many free-living species and those engaged in symbiotic associations with other plants and animals contribute significantly to the nitrogen fertility of aquatic and terrestrial habitats, including cultivated lands, particularly in the tropics (Fay,1983). Their usually high content of protein makes them prospective candidates for new unconventional sources of animal and human food. Indeed, in the central African state of Chad, dried cakes of *Spirulina*, which grows abundantly in soda lakes, constitute an important part of the diet of local people (Fay,1983). In the search for new sources of energy, some researchers consider cyanobacteria as the most promising agents for the development of a biological, solar energy conversion system, based on the concurrent light-driven generations of elemental O_2 and H_2 through the action of cyanobacteria. They also provide a relatively simple and useful model system for the study of fundamental cellular processes such as macromolecule synthesis, regulation of gene expression, cell differentiation and ordered

developmental pattern formation (Fay, 1983). Of great importance is their established role in the fundamental process of biological nitrogen fixation which is essential for the maintenance of the nitrogen status of the whole biosphere. Most of the cyanobacteria in the littoral zone are nitrogen fixing, and they make a significant contribution to the productivity of rocky shores and coral reefs (Mague and Holm-Hansen, 1975). On the whole, cyanobacteria do not represent an important part of the marine plankton. The major exception to this is the massive development of the nitrogen-fixing *Trichodesmium* (Taylor *et al.*, 1973) in certain tropical waters. In the process of nitrogen fixation atmospheric nitrogen (N_2) is reduced to ammonia (NH_3) as shown below in a reaction catalyzed by nitrogenase, a complex enzyme system; an absolute condition for this reaction is the absence of free O_2, since nitrogenase is inactivated in the presence of O_2 (Fay 1992).

$$N_2 + 8H^+ + 8e^- \xrightarrow{\text{nitrogenase}} 2NH_3 + H_2$$

Cyanobacteria are alone among the prokaryotic organisms which synthesize nitrogenase in evolving oxygen during photosynthesis, even though their nitrogen fixing enzyme, nitrogenase, is very sensitive to oxygen. There are three main types of morphology seen in nitrogen fixing cyanobacterial species. The first is those species which form heterocysts, and which fix nitrogen aerobically and which have been investigated most extensively e.g. *Anabaena cylindrica.* The second group comprises various non-heterocystous, filamentous forms, which are able to fix nitrogen micro- aerobically, that is in the presence of a little oxygen e.g. *Trichodesmium* (Fig.1.2). In the third group are unicellular, aerobic nitrogen-fixing species e.g. *Gloeothece* sp. (Fogg *et al.*,1973). Cyanobacteria that fix nitrogen and

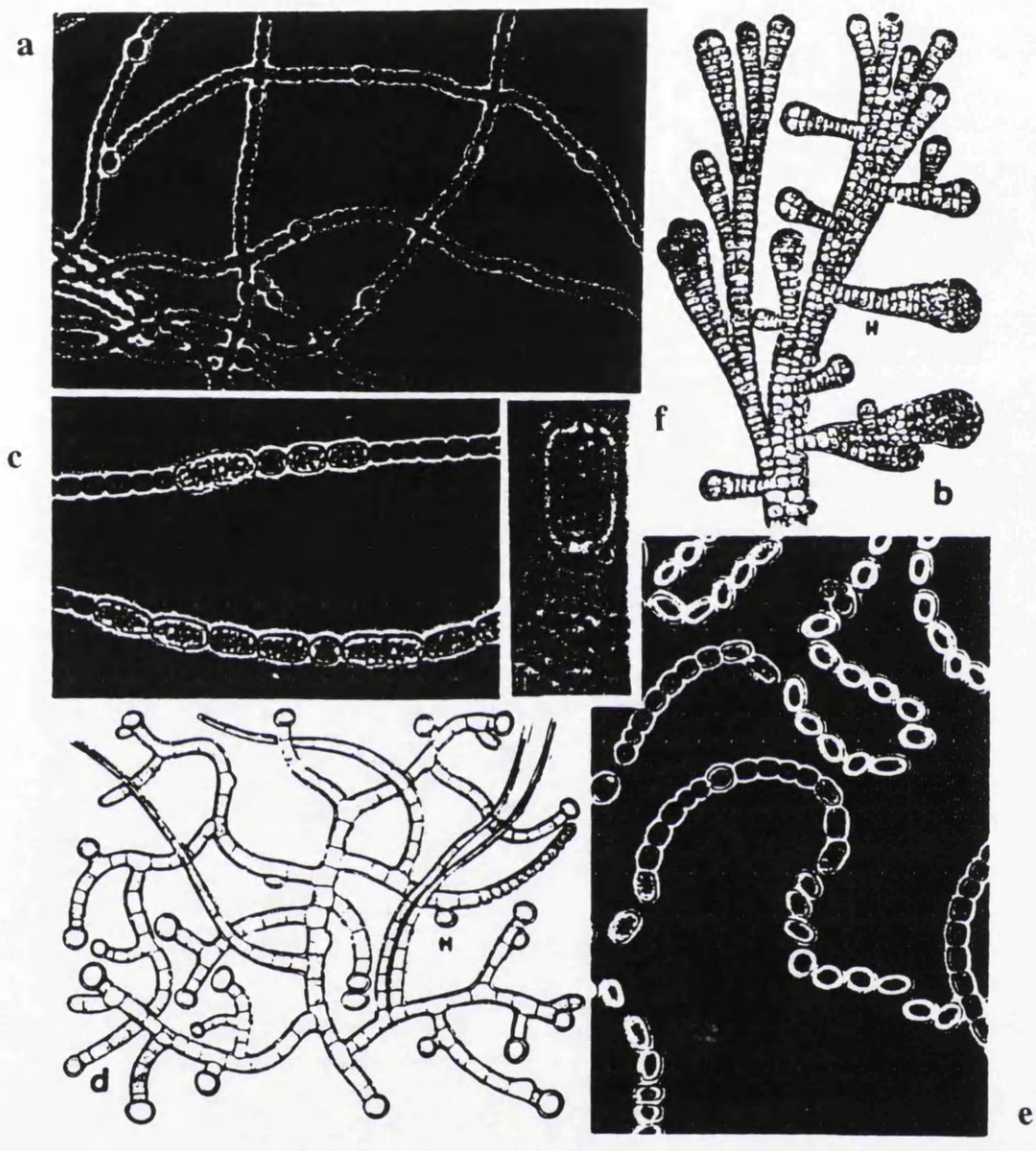


Fig 1.2 : Heterocyst - forming cyanobacteria. a. Anabaena variabilis; b. Hyphomorpha perrieri. c. Nostoc sp.; d. Mastigocoleus testarum. e. Anabaena cylindrica (Fogg strain); f. Detached heterocyst, and part of two vegetative cells, of Anabaena cylindrica strain ATCC 29414. A, akinete; H, heterocyst; P, proheterocyst; V, vegetative cell.(from Wolk *et al.*, 1994).

also produce oxygen must possess an efficient protective mechanism against oxygen inhibition of the enzyme nitrogenase. The role of heterocysts is to provide a source of fixed nitrogen for all cells in the filament, leaving to the other cells the task of acquiring carbon (Wolk, 1994). Heterocysts retain significant quantities of chlorophyll a and carotenoid pigments but essentially lack phycobiliproteins. Whilst heterocysts possess all the principal photosystem I components (Fay, 1983), much of the chlorophyll and phycobiliprotein associated with photosystem II units in vegetative cells is lost during heterocyst differentiation. Heterocysts are also devoid of the enzyme ribulose-1,5-bisphosphate carboxylase and of manganese (Mn^{2+}), an essential component of photosystem II. Heterocysts lack photosystem II of photosynthesis, and therefore, the oxygen-evolving capability, yet can form the necessary ATP for nitrogen fixation by cyclic photophosphorylation (Bottomley and Stewart, 1977; Tel-Or and Stewart, 1977). In contrast amongst those non-heterocystous cyanobacteria that are able to perform N_2 fixation under aerobic conditions, the first to be described was the unicellular *Gloeothece sp.* This cyanobacterium is able to grow photoautotrophically with atmospheric N_2 as sole N-source (Wyatt and Silvey, 1969) as it employs a temporal separation of oxygenic photosynthesis and nitrogenase activity (Khamees *et al.*, 1987).

Cyanobacteria not only have the ability to fix nitrogen, but they are also able to utilize both free CO_2 and bicarbonate ions as a source of inorganic carbon in photosynthesis (Fay 1983). The peripheral region of the cytoplasm contains the photosynthetic apparatus of cyanobacteria. In unicellular forms this commonly consists of a few membrane layers which extend in concentric sheets beneath the cell membrane and surround the central nucleoplasmic region. The more elaborate membrane structure seen in the cells of filamentous species is

apparently formed by means of expansion and invagination of the plasma membrane. The lipid bilayer of the thylakoid membrane incorporates the lipophilic photosynthetic pigments, chlorophyll a and the various carotenoids. It also incorporates the components of the electron transport chain including cytochromes, plastocyanin and ferredoxin (Fay, 1983). Although carboxylation of ribulose bisphosphate is the main route of CO_2 incorporation under optimum photosynthesizing conditions, the ribulose bisphosphate carboxylase pathway it is not the only route for CO_2-fixation. Carboxylation of phosphoenol pyruvate, catlayzed by the enzyme phosphoenol pyruvate carboxylase, represents another route for CO_2 uptake (Fig.1.3). Under limiting light conditions carbon assimilation is preferentially channelled towards the synthesis of amino acids and other essential cell consitutuents, but under saturating light quantities, sugars and starch are formed via the reductive pentose phosphate pathway. This indicates that at high illumination the rate of carbon fixation may exceed the rate of nitrogen assimilation, and hence the excess carbon and energy derived from photosynthesis is stored in the form of glycogen. Glycogen is the main reserve product which supports a limited dark metabolism and provides maintenance energy required for the essential cellular processes in the dark, through conversion to glucose-6-phosphate and metabolism via the respiratory pathways (Fay, 1983). Glucose-6-phosphate is oxidized and decarboxylated in two steps to ribulose-5-phosphate. The reactions are catalyzed by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, respectively. Both enzymes are present in high concentrations in cyanobacteria and both are NADP-specific: two molecules of NADPH are generated. The subsequent oxidation of NADPH during respiratory electron transport yields two molecules of ATP.

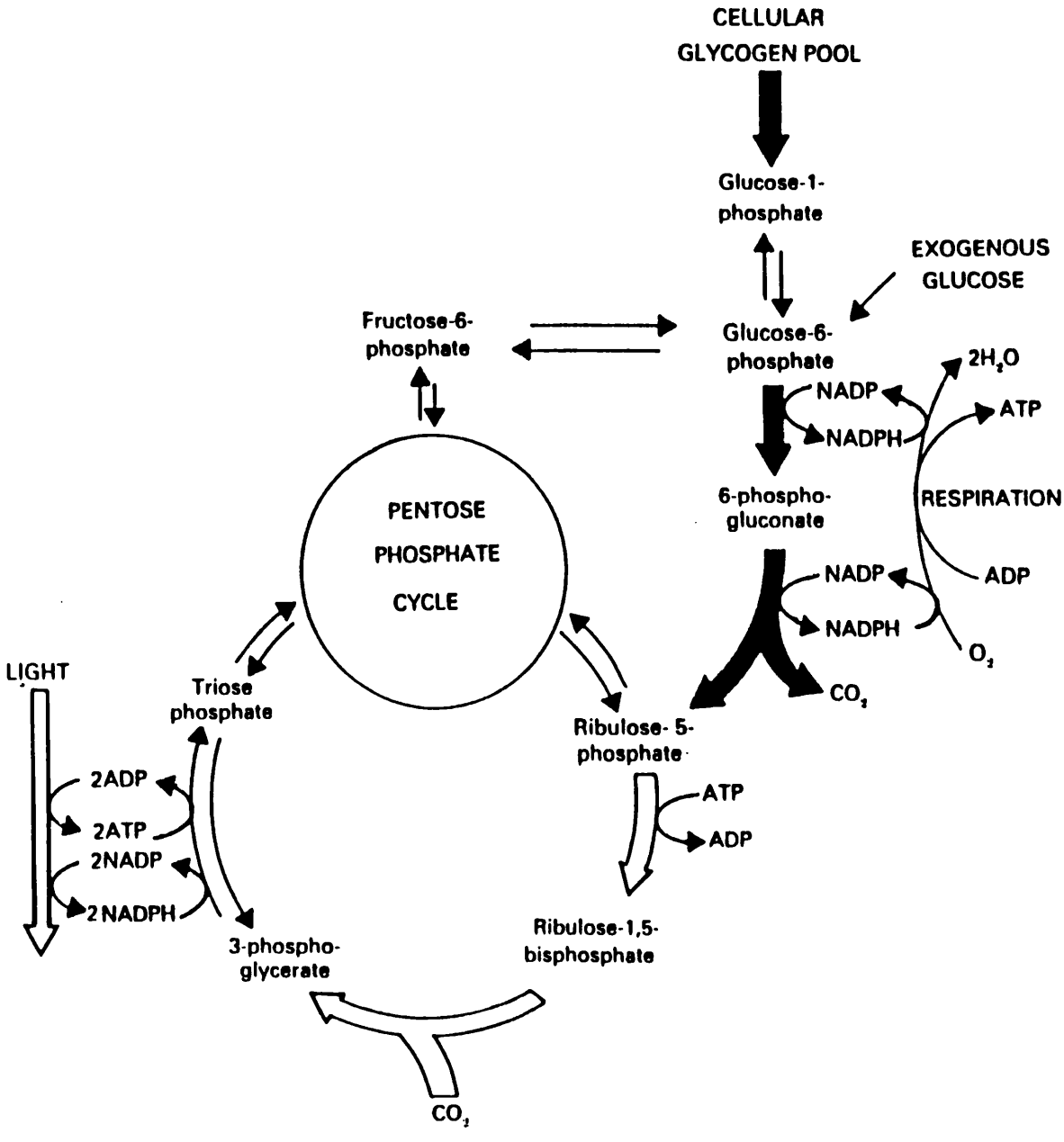


Fig 1.3 : Pathways of light and dark carbon metabolism in cyanobacteria. Reactions specific to photoautotrophic metabolism are indicated by white arrows, those specific to dark respiratory metabolism by black arrows (after Pelroy *et al.*, 1972, Arch. Microbiol., 87,303-22).

The oxidative pentose phosphate pathway is activated in the dark and inhibited in the light. Cyanobacteria, unlike aerobic heterotrophic bacteria and eukaryotic organisms, do not possess a complete tricarboxylic acid cycle (TCA). Some of the essential TCA cycle enzymes, like α-oxoglutarate dehydrogenase, succinyl-CoA-synthase and succinic dehydrogenase, are present only in extremely low concentrations or are absent from the cells (Fig.1.4). This deficiency prevents the cyclic flow of TCA cycle intermediates and consequently the utilization of substrates, such as acetate, pyruvate or tricarboxylic acids, for efficient energy production and growth in the dark. Though the incomplete TCA cycle does not function in substrate oxidation, it nevertheless performs a biosynthetic function enabling the synthesis of various amino acids. This pathway permits a limited flow of carbon from isocitrate to succinate and further toward the synthesis of porphyrins including chlorophyll, cytochromes and phycobiliproteins (Fay, 1983) .

The main factors which appear to determine the growth rate of cyanobacteria are light, temperature, pH , nutrient concentrations and the presence of organic solutes (Fay, 1983). One of the well-characterized phenomena associated with the acclimation of microorganisms to changes in ambient temperature is the regulation of the molecular motion or 'fluidity' of membrane lipids. A major mechanism of homeoviscous adaptation or temperature induced remodeling of membrane lipid is through changes in the extent of fatty acid unsaturation .

In response to lowered temperature bacteria which do not make polyunsaturated fatty acids change their fatty acyl lipid composition from saturated to monounsaturated fatty acids, whilst in species that synthesise fatty acids containing more than one double bond, such as most cyanobacteria, the content of polyunsaturated fatty acid is increased. In eubacteria membrane fluidity may also be

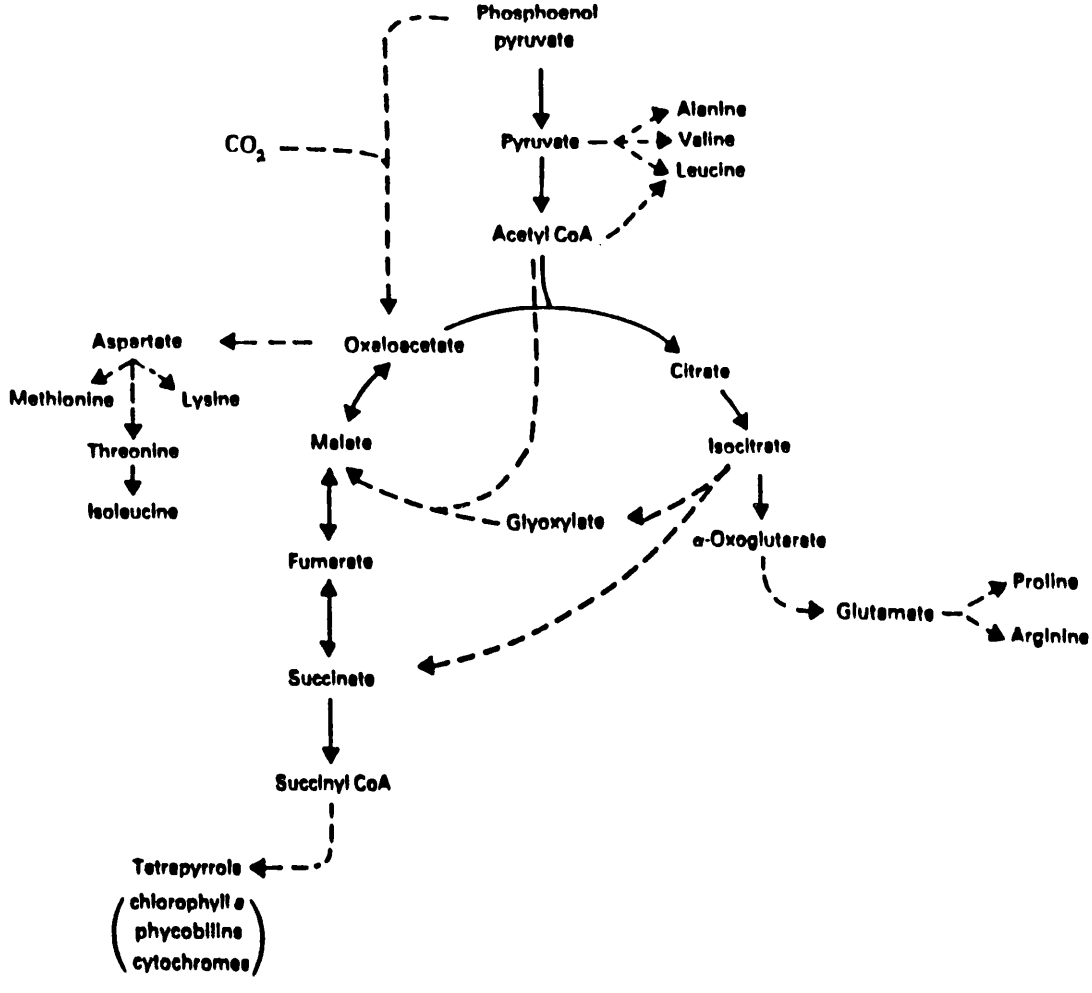


Fig.1.4 : The incomplete tricarboxylic acid cycle with the associated glyoxylate shunt and biosynthetic pathways in cyanobacteria (after Doolittle, 1979, Adv. Microb. Physiol.,20,1-102).

modified by shortening the average fatty acyl chain length, by increasing the amount of branched fatty acids or by decreasing the proportion of cyclic fatty acids (Russel 1984).

When organisms such as bacteria (Russel, 1984) and higher plants (Somerville and Browse, 1991) are exposed to temperatures below those of their normal growth conditions, an increase occurs in the degree of unsaturation of the fatty acids of their membrane lipids. These organisms can maintain the level of molecular motion of membrane lipids by regulating the number of double bonds in the fatty acids of these lipids (Cossins, 1994 ; Russel 1984). In response to a reduction in temperature which would be expected to result in a decrease in the fluidity of membrane lipids, cyanobacteria and plants introduce double bonds into the fatty acids of lipids, so that the membranes maintain a more fluid state (Jaworski, 1987; Harwood 1988).

In photosynthetic membranes, the relationship between the physical phase of membrane lipids and the temperature dependencies of physiological activities has been studied in chilling-sensitive higher plants (Somerville and Browse,1991; Nisahida and Murata, 1996), algae (Thompson, 1989; Nozawa and Umeki , 1988), bacteria (Russel, 1984), fungi (Miller and Baran, 1984) and animals (Stubbs and Smith, 1984). Effects of temperature are most evident as altered properties of the acyl chain domain in the bilayer interior. At physiological temperatures, *gauche* rotamers freely propagate up and down the length of the fatty acyl chains , which results in a relatively fluid, disordered liquid-crystalline phase (Mendelsohn *et al.*, 1989). However, acyl chain motion is moderately constrained for 8-10 carbon atoms extending from the membrane surface primarily by the covalent attachment and parallel alignment of the hydrocarbon chains (Collins

et al., 1990). When the temperature drops below the physiological range, acyl chains, at some defined point, adopt the all-*trans* conformation and pack efficiently to form a highly ordered gel phase (Fig. 1.5). However, in biological membranes, a region of phase separation may extend over a temperature range of 10-15° due to the diversity of lipid species present (Killian *et al* ., 1992). Conversely, when the temperature exceeds the physiological range, some lipids assume the inverted hexagonal phase (Fig. 1.5), which results in a loss of bilayer integrity (Mariani *et al.,* 1990; Seddon, 1990). The transition to the hexagonal phase is driven, in part, by a temperature-induced change in phospholipid molecular geometry from a cylindrical to a conical shape. Although 'conical' lipid can accommodate increased disorder in the acyl domain, it cannot alone form a lamellar or bilayer phase. Finally, even in the absence of lipid phase transition, rising temperature increases the rate and extent of acyl chain motion (Vrbjar *et al.*, 1992).

1.3 Cyanobacterial Pigments and Lipid Composition

The vegetative cyanobacterial cell is bounded by an envelope composed of the outer membrane and plasma (inner) membrane separated by a peptidoglycan layer, and contains intracellular photosynthetic membranes, termed the thylakoid membrane system. Their membrane structure is similar to that of the eukaryotic plant chloroplast, which also contains outer and inner envelope membranes, surrounding the thylakoid membranes. The cyanobacterial thylakoid membrane which is the site of both photosynthetic and respiratory electron transport (Omata and Murata, 1984b; 1985) contains chlorophyll-a and β-carotene (Omata and Murata, 1983; 1984a), and has phycobilisomes attached. (Gantt and Conti, 1969). The plasma

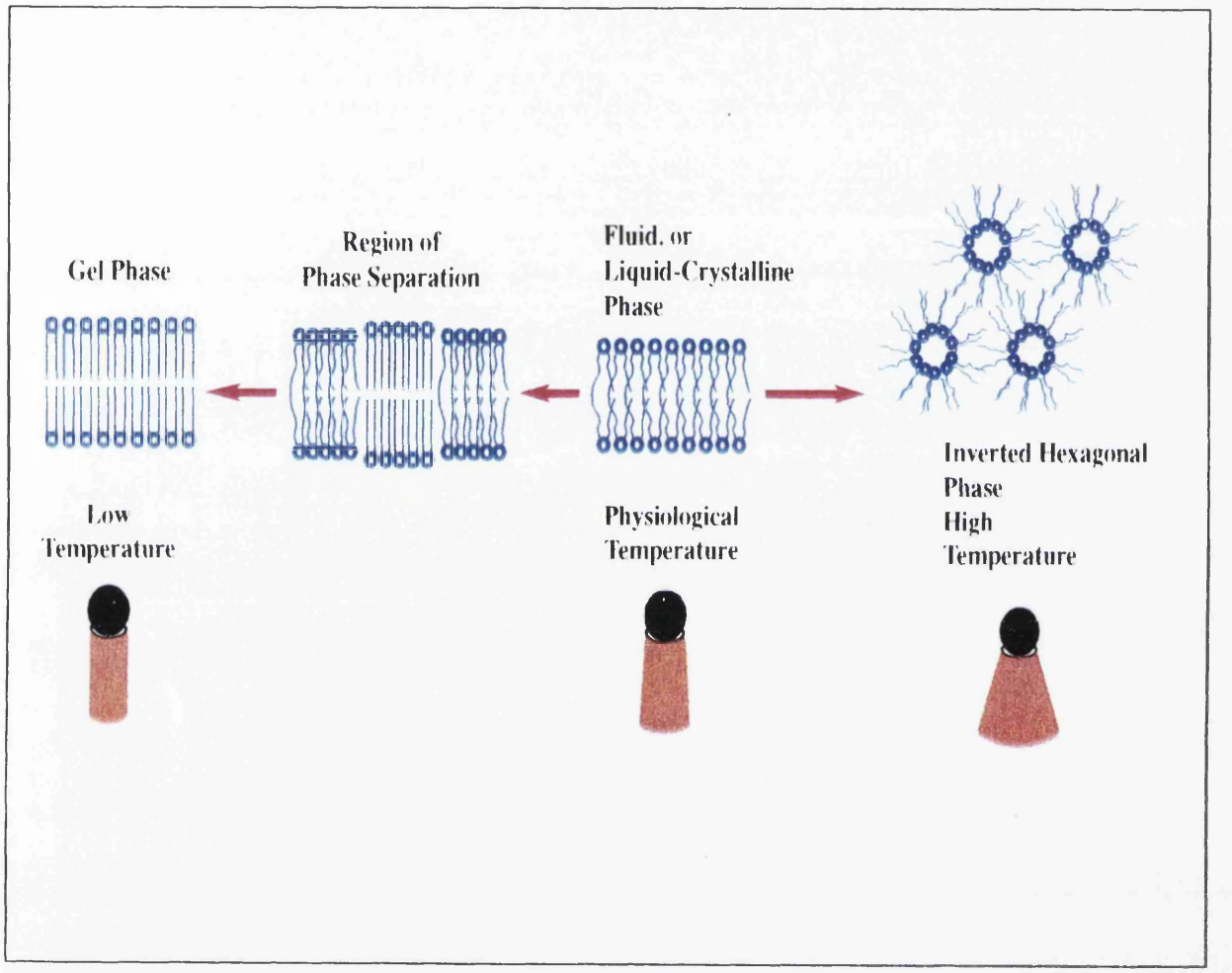


Fig 1.5 : Effect of temperature on membrane bilayer structure from Mendelsohn *et al.*, 1989

membrane and outer membrane typically contain xanthophylls (Table 1.1), but little or no chlorophyll-a or β-Carotene (Murata and Ono, 1981; Omata and Murata, 1983; 1984a; Resch and Gibson, 1983; Jurgens and Weckesser, 1985), and phycobiliosmes are never seen attached to these membranes (Stanier and Cohen-Bazire 1977). The thylakoid and plasma membranes contain glyceroglycolipids and phosphatidyl-glycerol (Nichols *et al.*, 1965), whereas the outer membrane contains lipopolysaccharides and hydrocarbons in addition to glycerolipids (Nichols and Wood, 1968b).

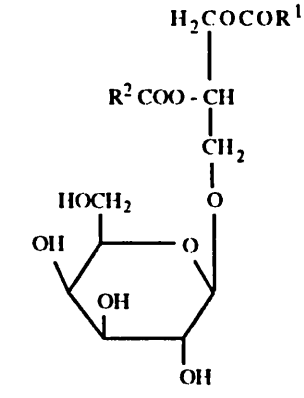
1.3.1 Glycerolipids

Cyanobacterial photosynthetic membranes contain four major lipid classes, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and phospatidylglycerol (PG) (Nichols *et al.*, 1965; Sato *et al.*, 1979, 1982a). Additional minor components including monoglucosyldiacylglycerol (GlcDG) (Feige *et al.*, 1980; Sato and Murata, 1982a) and trigalactosyldiacylglycerol (TGDG) are also present (Zepke *et al.*, 1978). The structures of these lipids are shown in (Fig 1.6).

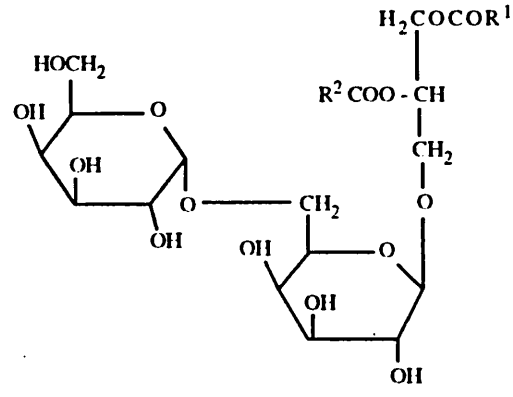
Cyanobacteria do not contain phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, diphosphatidylglycerol, or phosphatidylserine (Nichols *et al.*, 1965; Hirayama, 1967; Nichols and Wood, 1968a; Appleby *et al.*, 1971), which, in higher plants, occur predominantly in the extra-chloroplastidic membranes. MGDG typically contributes about half of the total cyanobacterial glycerolipids, and the other three glycerolipids contribute the remainder, the proportion of the individual lipids depending on the strains and being relatively independent of the growth conditions (Murata *et al.*, 1992; Murata and Nishida, 1987).

Table 1.1 The major photosynthetic pigments of cyanobacteria (Fay 1983).

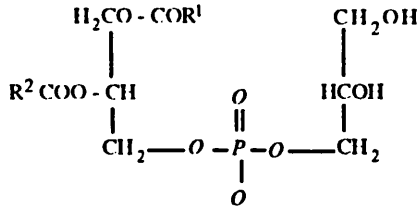
Group	Class	Major absorption bands (nm)
Chlorophylls	Chlorophyll a	435, (670), 680, (700)
Carotenoids	β-carotene	(431), 450-454, 478-480
	Echinenone	455-459, (475)
	Zeaxanthin	(430) 453, 479
	Canthaxanthin	466
	Myxoxanthophyll	504-508, 474-476, 450-452
Phycobiliproteins	Allophycocyanin	650
	Phycocyanin	610-625
	Phycoerythrin	555-565



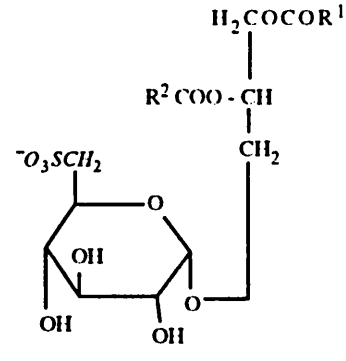
Monogalactosyl diacylglycerol
(MGDG)



Digalactosyl diacylglycerol
(DGDG)



Phosphatidyl glycerol
(PG)



Sulfoquinovosyl diacylglycerol
(SQDG)

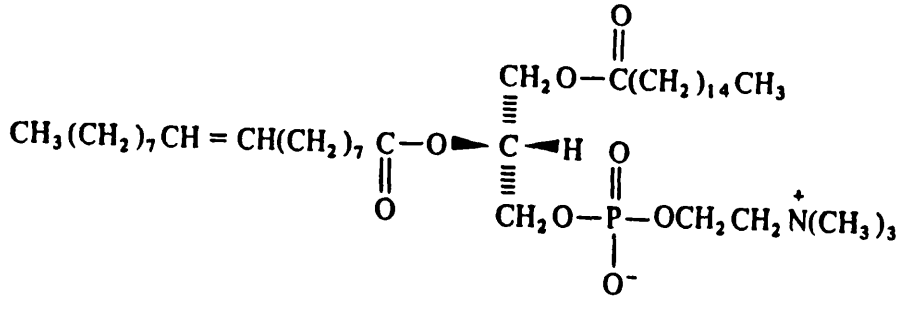
Fig 1.6 : The major lipid classes in cyanobacterial species. (Sato and Nishida , 1987).

Early studies of the fatty acid composition of glycerolipids from *Anacystis nidulans* (Holton *et al.*, 1964), and *Anabaena variabilis* (Levin *et al.*, 1964) established the fatty acids commonly found in cyanobacteria (Table 1.2). There are no branched chain fatty acids (Parker *et al.*, 1967) or Δ^3-*trans*-hexadecenoic acid found in cyanobacteria (Nichols *et al.*, 1965; Allen *et al.*, 1966; Nichols and Wood,1968a).

Each class of glycerolipid is a mixture of molecular species which contain different combinations of fatty acids. Each of the three glycerol hydroxyl groups may in principle be esterified with any fatty acid. There is , therefore, the possibility of an enormous number of glycerolipid species. To resolve this confusion the IUPAC-IUB Commission on Biochemical Nomenclature (1978) introduced the '*stereospecific numbering*' system. The carbon atom that appears at the top of the carbon chain when the glycerol molecule is written as a Fischer projection with the secondary hydroxyl group (i.e. that on the central carbon) to the left is designated C-1. To distinguish this numbering system from all others the prefix '*sn*' (for stereospecific numbering) is used. The application of this system to a glycerophospholipid is shown in Fig 1.7. The types of fatty acid in the *sn*-1 and *sn*-2 positions often have characteristic distribution. In animals, the pattern is usually *sn*-1-saturated, *sn*-2- unsaturated whilst in plants, in contrast there is no consistent pattern of acylation, there being differences between species and organelles (Russell, 1984). Analysis of glycerolipid molecular species from cyanobacteria has indicated that chain length determines the distribution in these organisms, with a characteristic *sn*-1-C_{18} and *sn*-2- C_{16} glycerol substitution pattern (Zepke *et al.*, 1978; Sato and Murata, 1980a). The most abundant molecular species in all the lipid classes of *Anabaena cylindrica*,

Table 1.2 Fatty acids commonly found in cyanobacteria

Common Name	Systematic Name	Abbreviations
Palmitic acid	Hexadecanoic acid	16:0
Palmitoleic acid	Hexadeca-9-*cis* enoic acid	16:1 (9)
_	Hexadecadienoic acid	16:2 (9,12)
Stearic acid	Octadecanoic acid	18:0
Oleic acid	Octadeca -9 *cis*- enoic acid	18:1 (9)
Linoleic acid	Octadeca-9,12-*cis*-dienoic acid	18:2 (9,12)
α-linolenic acid	Octadeca-9,12,15-*cis*-trienoic acid	18:3 (9,12,15)
γ-linolenic acid	Octadeca-6,9,12-*cis*-trienoic acid	18:3 (6,9,12)
	Octadecatetraenoic acid	18:4



(II) 1 - Palmityl-2-oleyl-sn-glycero-3-phosphocholine (or alternatively 3-sn-phosphatidylcholine)

Fig 1.7 : Application of the stereospecific numbering system to glycerol and a glycerophospholipid (adapted from Goodwin and Mercer 1985)

Oscillatoria chalybea, and *Nostoc calcicola* is α-18:3/16:0, and that of *Tolypothyrix tenuis* is 18:4/16:0 (Zepke *et al.*, 1978).

Understanding of the mode of desaturation of fatty acids in the membrane lipids of cyanobacteria was greatly improved by analysis of the composition of fatty acids, the distribution of fatty acids at the *sn*-1 and *sn*-2 positions of the glycerol moiety, and the position of double bonds in the fatty acids. Kenyon (1972) and Kenyon *et al.*, (1972) classified the cyanobacteria into four groups, as shown in Table 1.3, according to their fatty acid components and this classification has been more recently elaborated by Wada & Murata (1989) and Murata *et al.* (1992). Strains in the first group contain only saturated and monounsaturated fatty acids, such as 16:1 and 18:1, whereas strains in the other groups contain polyunsaturated fatty acids in addition to saturated and monounsaturated fatty acids. The second group is characterized by the presence of α-18:3, the third group by γ-18:3, whilst the fourth group is characterized by 18:4.

1.3.2 Heterocystous Glycolipids : structure and function

Certain filamentous cyanobacteria possess unique non-saponifiable glycolipids which have not been detected in any other class of plant (Nichols and Wood, 1968b). These glycolipids appear in filamentous heterocystous strains when they were grown under nitrogen fixing conditions (Walsby and Nichols, 1969; Wolk and Simon, 1969; Reddy, 1983) and have been reported to be formed in nitrogen-fixing unicellular strains grown under similar conditions (Lorch and Wolk, 1974).

The structures of the major heterocyst glycolipids of *Anabaena cylindrica* were determined as α/β glycosides of very long chain polyhydroxyalkanes and glycose esters of very long chain fatty acids (Bryce *et al.*, 1972 ; Lambein and Wolk,

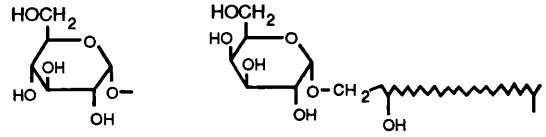
Table 1.3 Representative fatty acid distribution patterns found in cyanobacterial species

Class	Fatty Acids Present	Species	Reference
1	16:0, 16:1, 18:0, 18:1	*Anacystis nidulans*	Holton *et al.*, 1964 Levin *et al.*, 1964
2	16:0, 16:1, 16:2, 18:0, 18:1, 18:2, α-18:3	*Anabaena variabilis*	Rippka *et al.*, 1979
3	16:0, 16:1, 18:0, 18:1, 18:2, α-18:3, γ-18:3	*Spirulina platensis* *Synechocystis* Pcc6714	Murata *et al.*, 1992
4	16:0, 16;1, 18:0, 18:1, α-18:3, γ-18:3, 18:4	*Synechococcus* Pcc6803 *Tolypothrix tenuis*	Murata *et al.*, 1992

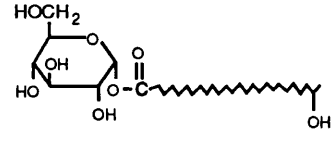
1973) as presented in Fig 1.8. Fractionation of heterocysts from *A.cylindrica* filaments established that this glycolipid fraction was found only in the heterocyst (Walsby and Nichols, 1969; Wolk and Simon, 1969; Reddy, 1983) where they form the laminated layer of the heterocyst envelope and can be termed heterocystous glycolipids (Walsby and Nichols 1969 ; Winkenbach *et al.*, 1972), where they may function in restricting entry of oxygen into the heterocyst (Walsby and Nichols, 1969). More recent studies have further established the presence of HG glycosides and glycose esters in other cyanobacteria, including a marine species (Soriente and Sodano, 1992)

1.3.3 Chlorophylls : structure and distribution in cyanobacteria

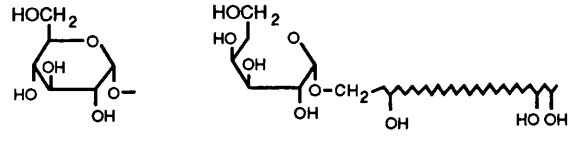
Chlorophyll, the essential catalyst of photosynthesis occurs universally as a green pigment in all photosynthetic plant tissues. It is bound loosely to protein and is easily extracted into lipid solvents such as acetone or ether. Chlorophyll contains a porphyrin (tetrapyrrole) nucleus with a chelated magnesium atom in the center and a long chain hydyrophobic phytyl side chain (Fig.1.9) esterified through the carboxylic acid group (Goodwin and Mercer, 1985). There are at least five chlorophylls in plants, distinguished by variations in the nature of the aliphatic side chains attached to the porphyrin nucleus; chlorophylls-a and b occur in higher plants, ferns and mosses, whilst chlorophylls-c to e are only found in algae (Vernon and Seely, 1966). In cyanobacteria however only chlorophyll a (Fig. 1.9) is present, and appears to be located exclusively in the thylakoid membrane in three different forms which can be distinguished on the basis of their absorption characteristics (maximum light absorption, λmax at 670, 680 and 700 nm, respectively) (Fay, 1983).



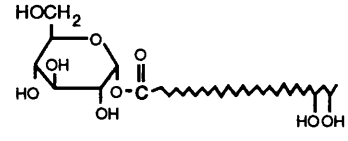
3,25-Dihydroxyhexacosanyl α-D-glycopyranoside



α-D-Glucopyranosyl
25-hydroxyhexacosanate



3,25,27-Trihydroxyoctacosanyl α-D-glycopyranoside



α-D-Glucopyranosyl
25,27-Dihydroxyoctacosanate

Fig.1.8 : Structures of heterocyst glycolipids (Bryce et al., 1972; Lambein and Wolk, 1973). The glycosyl ester glycolipids also contain lesser amounts of a-D-galactopyranosyl derivatives. *The positions of hydroxy groups on the hydrocarbon chain are tentatively determined (Lambein and Wolk, 1973).

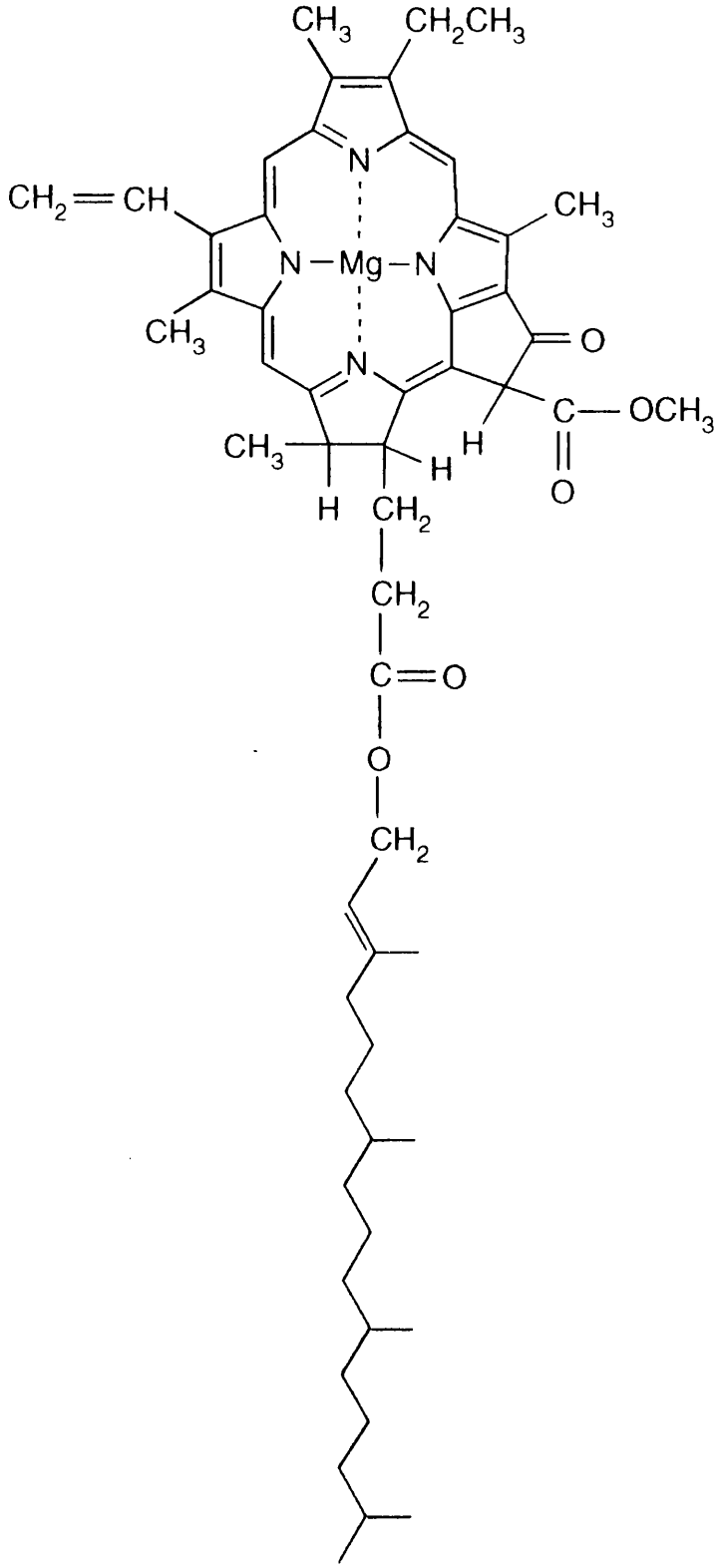


Fig1.9 : Chlorophyll a

1.3.4 Carotenoids : Structure and Distribution

The carotenoids in cyanobacteria occur predominantly in the lipid bilayers of the thylakoid membrane (Omata and Murata, 1983; 1984a), whilst the plasma membrane contains a small amount of β-carotene (Murata *et al.*, 1981: Omata and Murata, 1983;1984a). Among the carotenoids, β-Carotene appears to be universally present in all cyanobacteria while the presence and abundance of different xanthophylls (echinenone, zeaxanthin, oscillaxanthin, myxoxanthophyll) varies according to species (Table 1.1) (Fay, 1983). *Aphanizomenon* has also been reported to contain an unusual carotenoid aphanizophyll (4-hydroxymyxoxanthophyll) (Fig.1.10). Yamamoto and Bangham (1978) observed that the xanthophyll zeaxanthin incorporated into phospholipid liposomes exhibited a characteristic absorption change when the liposome membrane goes from a liquid crystalline to a phase separation state, and a very similar absorption change can be measured in intact cells and extracted lipids of *Anacystis nidulans* exposed to low temperature (Brand, 1977, 1979; Ono and Murata, 1981a). The spectral change is observed predominantly in the plasma membrane preparation in which zeaxanthin amounts to 70% of the total carotenoids, but little change is observed in the thylakoid membrane preparation in which β-Carotene is the major carotenoid component (Omata and Murata, 1983).

1.4 Glycerolipid Biosynthesis and Metabolism in Cyanobacteria

1.4.1 *De novo* Fatty Acid Biosynthesis

The general pathway for *de novo* biosynthesis of fatty acids is shown in Fig. 1.11. All organisms that synthesise fatty acids *de novo* require two enzymes: acetyl-

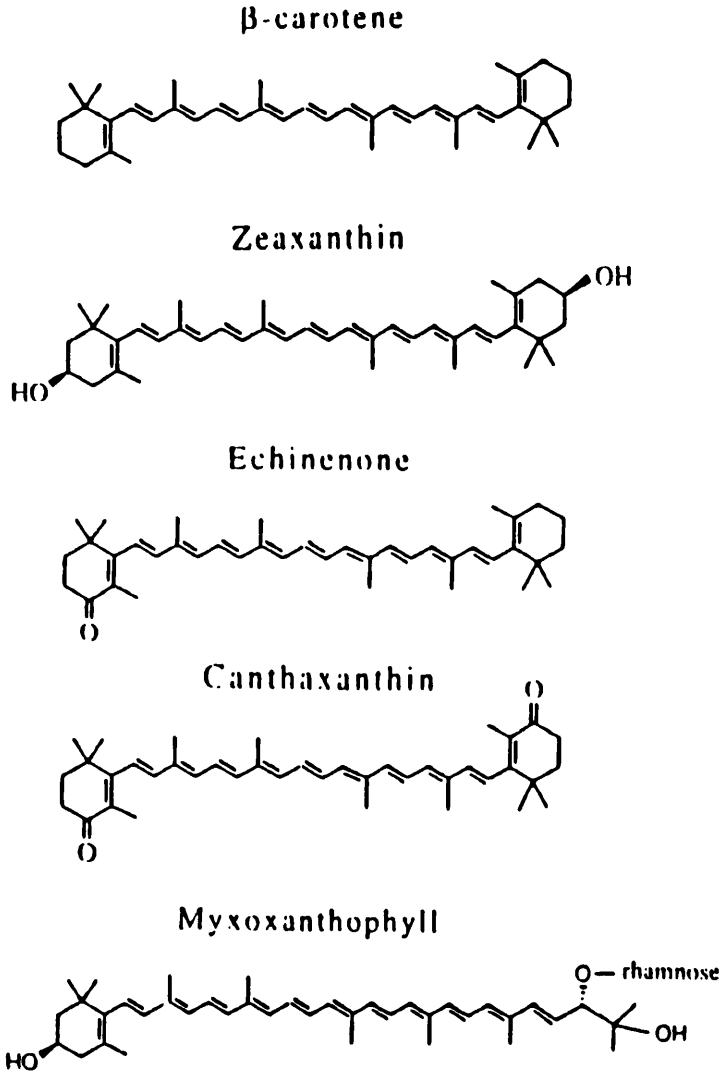


Fig 1.10 : Chemical structures of carotenoids common in cyanobacteria.

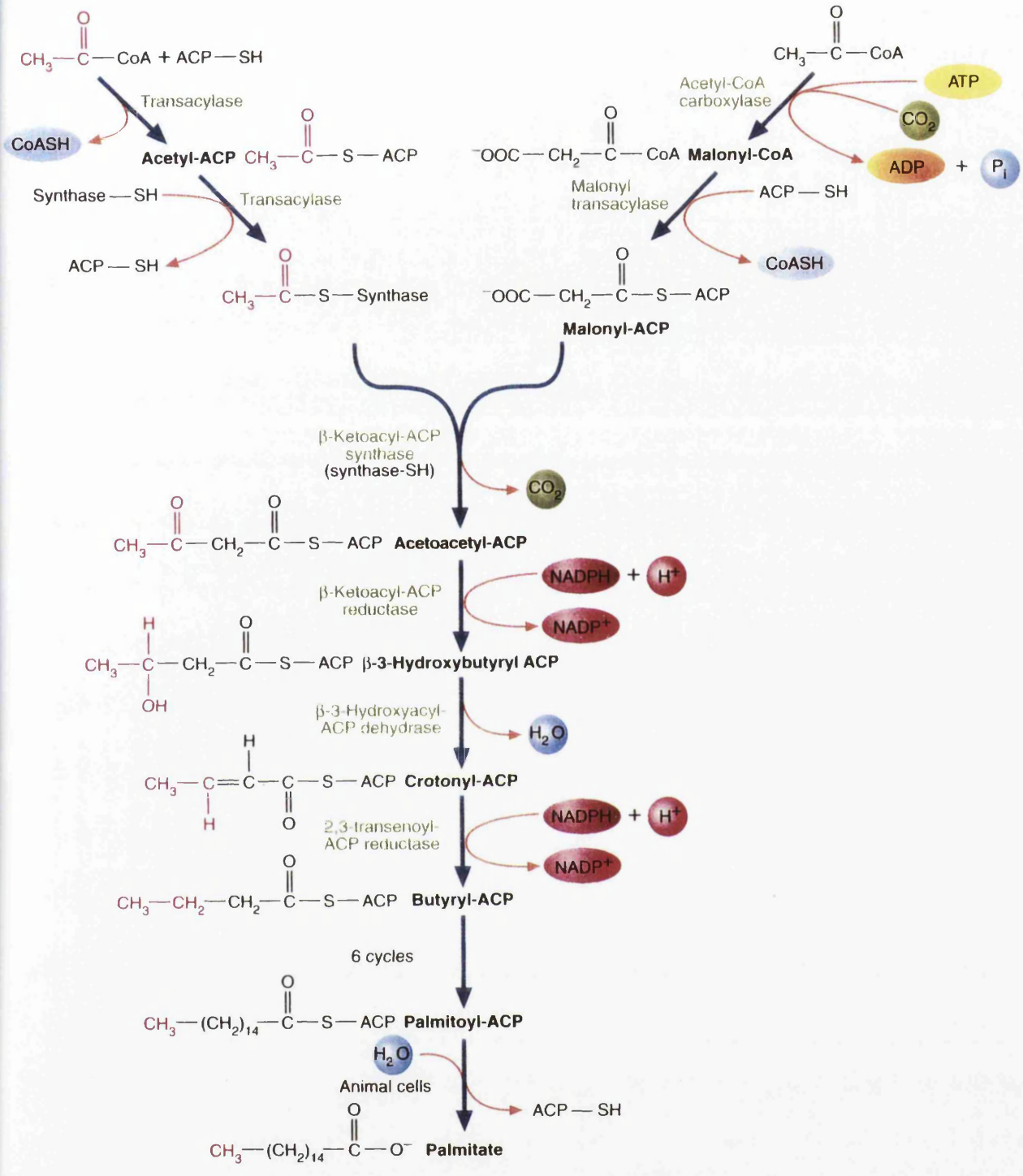


Fig. 1.11 Fatty acid biosynthesis general pathway for *de novo*.

CoA carboxylase (ACCase) and fatty acid synthase (FAS). ACCase is a biotin-containing enzyme that catalyse the following overall reaction:

$$MgATP + HCO_3^- + \text{acetyl-CoA} \longrightarrow MgADP + Pi + \text{malonyl-CoA}$$

Different isoformes of acetyl CoA carboxylase have been described recently in higher plants. Monocots appeare to contain at least two forms multifunctional proteins with acetyl CoA carboxylase activety (Herbert *et al.*, 1994), whilst dicots contain a multienzyme complex form of the enzyme in their chloroplasts and a multifunctional protein isoform in their cytosol (Alban *et al.*, 1994). This carboxylation reaction, which is the first committed step in *de novo* fatty acid biosynthesis, proceeds in two distinct half- reactions (Knowles, 1989). In the first step a biotin moiety that is covalently attached to the enzyme via a specific lysine residue is carboxylated in a MgATP- dependent manner.

$$\text{E-biotin} + MgATP + HCO_3^- \longrightarrow \text{E-biotin-}CO_2^- + MgADP + Pi$$

The carboxyl group is subseqently transferred from carboxybiotin to acetyl-CoA to form malonyl-CoA as shown in the following reaction (Alban *et al.*, 1995)

$$\text{E-biotin-}CO_2^- + CH_3\,CO.SCoA \longrightarrow \text{E-biotin} + HOOC.CH_2COSCoA$$

acetyl-CoA malonyl CoA

It has been proposed that ACCase from cyanobacteria or the chloroplast isoform from higher plants also play regulatory roles in fatty acid biosynthesis (Sasaki *et al.*, 1993; Alban *et al.*, 1994; Konishi and Saski, 1994). FAS transfers the malonyl

moiety to acyl carrier protein (ACP) and catalyzes the extension of the growing acyl chain with malonyl-ACP (Ohlrogge and Jaworski, 1997). In cyanobacteria and in the chloroplasts of higher plants saturated fatty acids are synthesized by dissociable fatty acid synthases (Harwood, 1980, 1996) as compared with the multifunctional protein complexes which constitute the FAS systems located in the cytosol of animal and fungal cells (Schweizer *et al.*, 1978; Sukamoto *et al.*, 1983). In plants and bacteria the initial FAS reaction is catalyzed by 3-ketoacyl ACP-synthetase (KAS III, Fig 1.12), which results in the condensation of acetyl-CoA and malonyl-ACP (Jaworski *et al.*, 1993 ; Topfer *et al.*, 1995). In the condensation reaction, a four carbon unit is formed from a two carbon unit and a three carbon unit, and CO_2 is released. Before a subsequent cycle of fatty acid synthesis begins, the 3-ketoacyl-ACP intermediate is reduced to the saturated acyl-ACP in the remaining FAS reactions (Fig. 1.11), catalyzed by the sequential activity of the 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydrase, and the enoyl- ACP reductase. In both plants and bacteria the final product of FAS is usually regarded as palmitoyl-ACP (16:0-ACP), which is then elongated through the activity of a C_{16}-specific 3- ketoacyl ACP synthase (KAS II Fig. 1.12) to stearoyl-ACP (18:0-ACP). In cyanobacterial cells, 16:0-ACP and 18:0-ACP are produced by the fatty acid synthase system (Lem and Stumpf, 1984a; Stapleton and Jaworski, 1984b), and the fatty acids synthesis mechanisms are similar to those in the cells of higher plants (Murata and Nishida,1987). Neither acyl-ACP desaturase nor acyl-ACP hydrolase has been found in cyanobacterial cells. The acyl groups of the saturated fatty acyl-ACPs are directly incorporated into glycerolipids by acyltransferases and then converted to unsaturated fatty acids by acyl-lipid desaturases (Murata and Wada, 1995) which utilize fatty acids already in lipid-boun

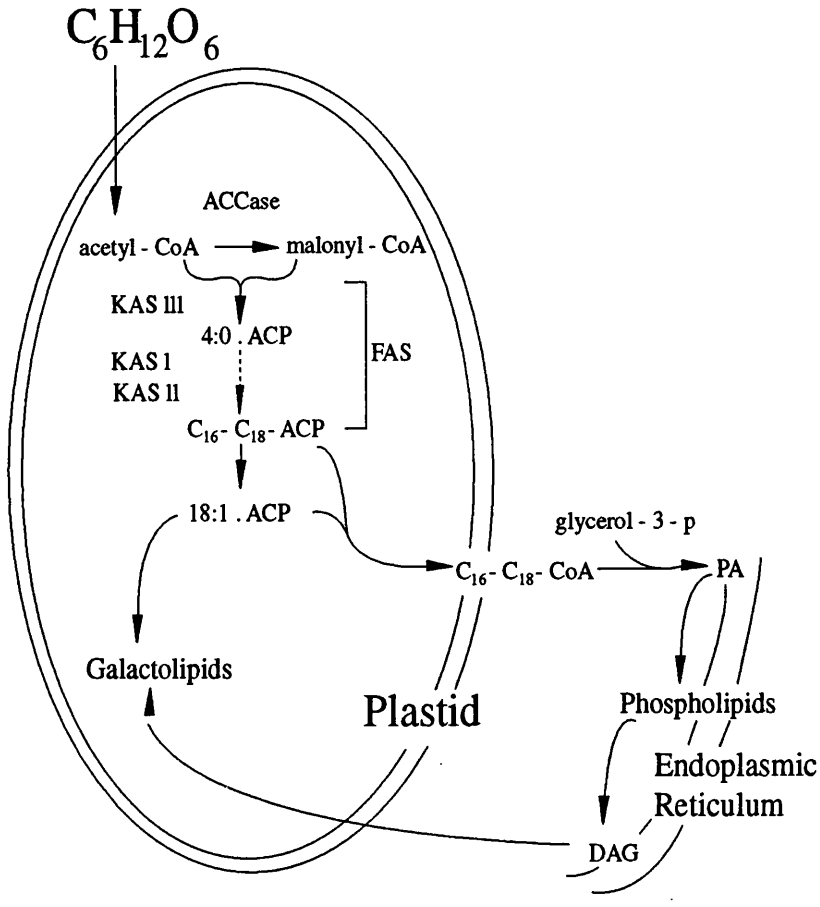


Fig 1.12 : Simplified schematic of overall flow of carbon through fatty acid and lipid metabolismin a generalized plant leaf cell (modified from Ohlrogge and Jaworski,1997).

form as substrates. This situation is different from that in higher plants where the first double bond is introduced while the fatty acid is bound to acyl-carrier (ACP) protein (Mckeon and Stumpf 1982; Stumpf 1981) whilst subsequent desaturations utilize an acylglycerolipid substrate. In contrast unsaturated fatty acid synthesis in cyanobacteria is characterized by acyl-lipid desaturation in each desaturation reaction (Sato & Murata, 1982a; Sato *et al.*, 1986). In all cases, the double bonds are invariably located at exact positions in the fatty acid methyl ester chain. For example in desaturation of C-18- fatty acids in Group 2 cyanobacteria, the first double bond is introduced exclusively at the Δ^9 position, and the subseqent desaturations occur at the Δ^{12} and the Δ^{15} positions. Whilst, in cyanobacterial strains in Groups 3 and 4, a double bond is introduced at Δ^6 position (Murata *et al.*, 1992). This suggests that the desaturases can count the exact number of carbon atoms in the hydrocarbon chain and can introduce the double bond at a specific position (Fig 1.13). Another interesting characteristic of lipid synthesis in cyanobacteria is the specific distribution of fatty acids at the *sn* positions of the glycerol moiety of glycerolipids. In the cyanobacterial strains in groups 2, 3, and 4 the C-18- fatty acids are esterified at the *sn*-1 position whilst the C-16- fatty acids are esterified at the *sn*-2 position (Murata *et al*, 1992). This suggests that the acyl ACP: glycerol-3-P-acyltransferase uses 18:0-ACP specifically as the acyl-donor substrate and that acyl-ACP: 1-acylglycerol-3-P-acyltransferase is specific for 16:0-ACP.

1.4.2 Formation of Glycerolipids

In cyanobacteria, which lack both phosphatidylcholine and a chloroplast (Appleby *et al.*, 1971), the pathway of biosynthesis of MGDG is necessarily different

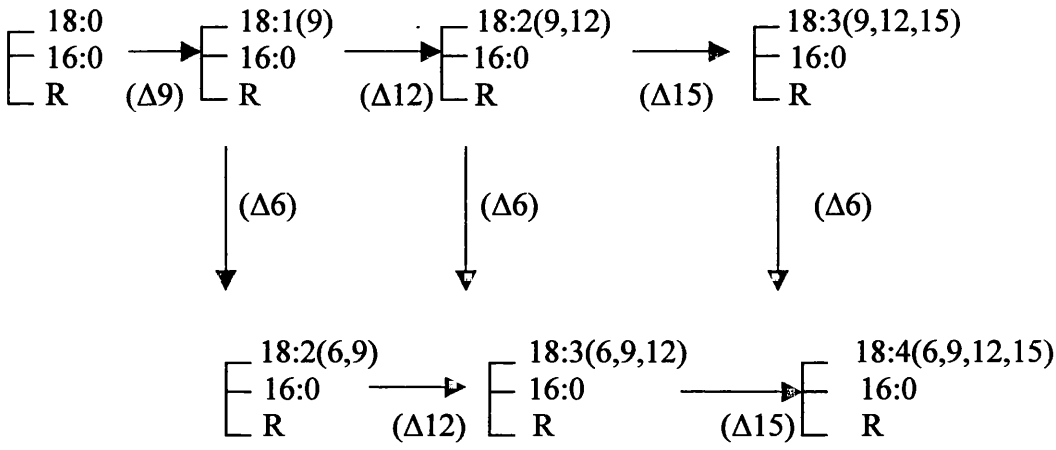


Fig 1.13 The pathway of fatty acid desaturation by acyl-lipid desaturase in *Synechocystis* sp. PCC 6803. Fatty acids are represented by X:Y (Z) in which X and Y indicate, respectively, the number of X carbon atoms and the number Y double bonds in the *cis* configuration at the Z position counted from the carboxy terminus e.g.18:2 (9,12) represents an C-18 acid with 2 *cis* double bonds of positions 9 and 12. R= polar lipid head group.(after Higashi and Murata,1993).

from that in the higher plants. In higher plants MGDG is synthesized in the chloroplast envelope by transformation of phosphatidylcholine produced in the endoplasmic reticulum (Roughan *et al* 1980; Simposn & Williams 1979; Douce and Joyard 1982) and DGDG is synthesized from MGDG by dismutation of two molecules of MGDG (Van Besouw and Wintermans, 1978) or by transfer of a second galactose unit to MGDG (Ongun and Mudd, 1968 ; Siebertz and Heinz, 1977).

In studies of cyanobacterial glycolipid biosynthesis however Appleby *et al.*, (1971) and Nichols (1968) found that [^{14}C]-acetate was incorporated into the four classes of glycerolipid lipids, and that the ^{14}C-distribution remained essentially constant during incubation periods from 45 min to 24 h. Subsequently Feige *et al.* (1980) conducted $H^{14}CO_3$ pulse-labeling experiments in 30 species of cyanobacteria, and their observation that ^{14}C is rapidly incorporated into GlcDG, then subsequently appears in MGDG, led to the proposal that GlcDG is a precursor of MGDG in the biosynthesis of glycolipids in the cyanobacteria. This was subsequently confirmed by Sato and Murata (1982a) who studied the pathway of glycolipid biosynthesis in *A. variabilis*, in which the content of GlcDG, at about 1% of the total lipid, is much higher than that in other cyanobacterial species (usually less than 0.1% of the total lipid) (Feige *et al.*, 1980). They found that the primary product is GlcDG, which is converted to MGDG.

There are two possible mechanisms for the conversion of GlcDG to MGDG. The first is a replacement of the glucose unit by a newly synthesized galactose unit, and the second is epimerization at the C-4 atom of the glucose unit initially transferred. Sato and Murata (1982a) showed in pulse-labeling studies that the radioactivity in the sugar moiety remained almost constant during chase experiments,

supporting the conclusion that in *A. variablilis* the conversion from GlcDG to MGDG results from epimerization of glucose to galactose - that is, stereochemical isomerization at the C-4 atom of the glucose unit in the glycerolipid and not by replacement of glucose by galactose. Sato and Murata (1982a) also demonstrated that DGDG is produced by transfer of newly synthesized galactose from uridine diphosphate galactose (UDP-galactose) to MGDG, and that SQDG and PG are also rapidly labeled, suggesting that the latter lipids are directly synthesized by pathways that do not involve GlcDG. Subsequently Sato and Murata (1982b) demonstrated a membrane-associated UDP-glucose:diacylglycerol glucosyltransferase activity in preparations from *A. variabilis* which transfered glucose from UDP-glucose to diacylglycerol to produce GlcDG. Similar GlcDG synthesis activity appeared to be located in both the thylakoid and plasma membranes of *Anacystis nidulans* (Omata and Murata, 1986). Interestingly, in higher plant chloroplasts the activity of galactolipid synthesis is confined to the envelope membranes (Douce and Joyard, 1979), although, in etioplasts of wheat seedlings, the galactoslytransferase activity is detected in both envelope and pro-thylakoid membranes (Sandelius and Selstam, 1984). Two mechanisms have been proposed for the synthesis of DGDG in higher plants. Ongun and Mudd (1968); Siebertz and Heinz (1977) have inferred that uridine diphosphate galactose (UDP-galactose) donates the galactose unit to MGDG to form DGDG, whilst Van Besouw and Wintermans (1978), have presented evidence consistent with the dismutation of two molecules of MGDG to produce one molecule of DGDG and one molecule of diacylglycerol.

The activities of glycerol-3-phosphate acyltransferase and 1-acylglycerol-3-phosphate acyltransferase, the enzymes involved in the first steps of glycerolipid synthesis, have been characterized in crude extracts of *A. variabilis* (Lem and

Stumpf, 1984). These enzymes used acyl-ACP, but not acyl-coenzyme A as the substrate. These observations indicate that the precursor of the glycerolipids is phosphatidic acid, which is converted to the glycerolglycolipids and glycophospholipids in this cyanobacterium. Interestingly, the crude extract of *A. variabilis* is active in transferring 18:1 from 18:1-ACP to glycerol-3-phosphate, although, in contrast to the case of higher plants, 18:1 is not synthesized in the ACP-bound form in this cyanobacterium (Lem and Stumpf, 1984).

1.4.3 Biosynthesis of Unsaturated Fatty Acids in Cyanobacteria : Isotopic Labelling Studies

Fatty acid desaturation has been most extensively studied in *A. variabilis*, which contains polyunsaturated fatty acids (Table 1.3). After 6h labeling of the cyanobacterial cells with [2-^{14}C]-acetate, Appleby and co-workers (1971) observed that radioactivity was incorporated into 18:0 (5%), 18:1 (52%), 18:2 (13%), 16:0 (25%), and 16:1 (6%). Externally added [1-^{14}C] 18:1 was also incorporated into the glycerolipids, and a small proportion of the 18:1 was also converted to 18:2 (Appleby *et al.*, 1971). Sato and Murata (1982b) showed, by pulse-labeling for 6 min with $H^{14}CO^{-}_{3}$, that radioactivity is incorporated into 16:0, 18:0 and 18:1 of GlcDG, SQDG, and PG. An extension of labeling time up to 1 h resulted in an increase in radioactivity of 18:1 relative to that of the total fatty acids and corresponding decrease in that of 18:0 (Sato and Murata 1982b). These changes in radioactivity associated with 18:0 and 18:1 proceeded further during a subsequent chase for 10h. It was therefore suggested that, in *A. variabilis*, only saturated fatty acids (i.e., 16:0 and 18:0) are first esterified to the lipids and that these are then desaturated whilst they are bound to the glycerol moiety. The lipid-linked

desaturation of 16:0 to 16:1 in MGDG was confirmed by a combination of $H^{13}CO^{-}_3$ feeding and mass spectrometric analysis of the 2-acylglycerol moiety of ^{13}C-enriched MGDG molecular species (Sato *et al.*, 1986).

1.4.4 Fatty Acid Desaturase Systems in Cyanobacteria

Saturated fatty acids are converted to unsaturated fatty acids in reactions catalyzed by fatty acid desaturases, which are enzymes that convert a single bond between two carbon atoms to a double bond in a fatty acyl chain (Harwood, 1980; Stumpf, 1980); the resultant double bond is often referred to as an unsaturation. As shown in Fig. 1.14 three types of fatty acid desaturase, distinguished by use of acyl-CoA, acyl-ACP or acyl-glycerolipid as substrate have been described (Murata and Wada, 1995). These reactions require molecular oxygen and occur under aerobic conditions (Jaworski, 1987; Stumpf, 1980). In cyanobacteria, for example *A. variablis*, stearic and palmitic acid are initially esterified, respectively, to the C-1 and C-2 positions of the glycerol moiety of MGDG, and then desaturated whilst still attached to the polar lipid (Sato *et al.*, 1979; Sato and Murata, 1980b; 1981; 1982b).

In cyanobacteria and hight plants most acyl-lipid desaturases consist of 300-350 amino acid residues, being hydrophobic proteins that appear to span the membrane four times (Murata and Wada, 1995). Such enzymes in cyanobacterial cells and chloroplasts use ferredoxin as the electron donor (Wada and Murata, 1993; Wada *et al.*, 1993). With the exception of the soluble Δ^9 desaturase of higher plant chloroplast they are bound to the endoplasmic reticulum or the thylakoid chloroplast membrane in plant cells (Jaworski, 1987), and the thylakoid membrane in cyanobacterial cells (Wada *et al.*, 1993). The cyanobacterium, *Synechocystis sp. Pcc 6803*, has four acyl-lipid desaturases (Murata and Wada, 1995). These enzymes

(i) **Acyl-CoA desaturase**

18:0-CoA → 18: 1(9) – CoA → 18: 2 (6,9)-CoA

(ii) **Acyl-ACP desaturase**

18:0-ACP → 18: 1(9))-ACP

(iii) **Acyl-lipid desaturase**

(a) **In plants**

18: 1 (9)		18: 2(9, 12)		18: 3 (9, 12, 15)
16:0	→	16: 0	→	16:0
X		X		X

(b) **In cyanobacteria**

18: 0		18: 1(9)		18: 2(9, 12)		18: 3(6, 9, 12)		18: 4(6, 9, 12, 15)
16: 0	→	16: 0	→	16: 0	→	16: 0	→	16: 0
X		X		X		X		X

Fig 1.14 The three types of fatty acid desaturase, with typical examples of desaturation reactions (Murata and Wada, 1995) X represents a polar head group.

termed des C, des A, des D, and des B, catalyse desaturation at the Δ^9, Δ^{12}, Δ^{15} and Δ^6 positions, respectively, of fatty acids esterified at the *sn*-1 position of the glycerol moiety of glycerolipids (Murata *et al.*, 1992; Higashi and Murata, 1993; Murata and Wada, 1995). The Δ^9 (Sakomoto *et al.*, 1994, Sakamoto and Bryant, 1997) desaturase introduces the first unsaturated bond into lipid-bound stearic acid to produce oleic acid, which is further desaturated to linoleic acid by the Δ^{12} desaturase (Sakamoto and Bryant, 1997; Wada, *et al.*, 1990). The Δ^6 (Reddy *et al.,* 1993) and Δ^{15} (Sakamoto *et al.*, 1994) desaturases introduce further unsaturated bonds to generate trienoic and tetraenoic fatty acids as shown in Fig 1.14.

The specific site of desaturation by these enzymes is defined by reference to the carboxyl terminus (C-1) position or the methyl terminus (ω -position) of the fatty acid (Higoshi and Murata, 1993; Murata *et al.,* 1995). The Δ^{12} and Δ^6 desaturases introduce a double bond into lipid-bound fatty acids that have a double bond at the Δ^9 position (Murata and Wada, 1995), whilst the Δ^{15} desaturases show specificity for fatty acids that have a double bond at the Δ^{12} position (Higashi and Murata, 1993).

All known desaturases are characterized by the presence of three histidine clusters, which are localized at strongly conserved positions in the amino acid sequence of each protein (Murata & Wada, 1995; Shanklin and Cahoon 1998). It has been suggested that these clusters are involved in the formation of the active site of each desaturase, as has been demonstrated in other di-iron enzymes (Fox *et al.*, 1993, 1994; Shanklin *et al.*, 1994).

It is of considerable interest that the histidine clusters in the Δ^9 acyl-lipid desaturases from cyanobacteria are identical to those in Δ^9 acyl-CoA desaturases from animal, yeast and fungal cells. Moreover, the details of histidine clusters in cyanobacterial desaturases are specific to the type of desaturase, when the

desaturases are classified with respect to the position in the acyl chain at which each introduces an unsaturated bond. Fig 1.15 shows the predicted positioning of the Δ^{12} acyl-lipid desaturase in relation to the membrane. The enzyme spans the membrane four times and exposes the three histidine clusters to the cytoplasmic acid (Los *and* Murata, 1998).

1.5 Lipid Retailoring During Temperature Adaptation in Cyanobacteria

Temperature is one of the most important environmental factors that influence the fatty acid composition of membrane lipid (Sumner *et al.*, 1969; Hazel and Prosser, 1974). Cossins (1977, 1990) illustrated that living organisms, in particular poikilotherms, respond to a downward shift in temperature by desaturating the fatty acids of their membrane lipids in a process termed homeoviscous adaptation. This acclimatizing response reflects the organism's ability to maintain the fluidity of cellular membranes over a certain range of temperatures. The temperature effects on fatty acid and membrane lipid molecular species composition are studied in two ways; (i) analysis of the fatty acid composition in organisms grown isothermally at several temperatures, and (ii) study of the changes in fatty acid composition consequent to a growth-temperature shift. (Okuyama *et al.*, 1977; Nozawa and Kasai, 1978). These changes in fatty acid composition are interpreted in terms of the regulation of membrane fluidity for the proper function of biological membranes (Cronan and Gelmann, 1975; Thompson and Nozawa, 1977). Consequently, to function over a broad range of environmental temperatures poikilothermic organisms must restructure their membranes so that lipids of appropriate physical properties are matched to prevailing thermal conditions.

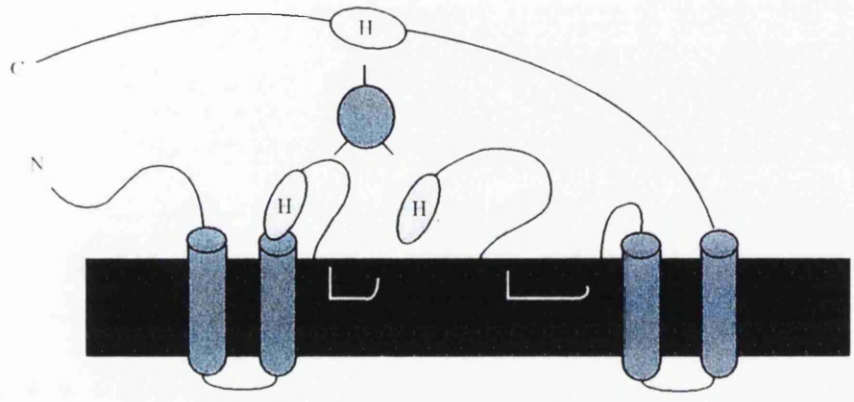


Fig 1.15: The predicted positioning of the Δ 12 acyl-lipid desaturase in the membrane: a computer prediction based on the localization of the conserved histidine clusters and iron atoms, which are assumed to constitute the catalytic center, at the cytoplasmic side (from Los *et al.*,1998).

H = histidine cluster
= membrane - spanning desaturase domain
= fatty acid chain

Accordingly, the most commonly observed cellular response to altered temperature is a remodeling of biological membranes. Compositional studies of cyanobacteria have demonstrated that shifts in growth temperature lead to several types of changes in lipid composition. Growth at low temperature, for example, causes one or more changes in membrane lipid composition, including increases in fatty acid unsaturation, changes in the proportions of lipid classes and changes in the lipid: protein ratio (Cossins,1994a). Each of these changes leads to membrane remodelling and to alterations in the organization and dynamic properties of membrane lipids. Lowering growth temperature led to an increase in fatty acid unsaturation and shortening of acyl chain length (Holton *et al.*, 1964; Sato *et al.*, 1979; Suutari and Laakso, 1990, 1999). The glycerolipids of *A. nidulans,* a Group 1 cyanobacterium, contain fatty acids with 14, 16 and 18 carbon atoms; these fatty acids are distributed on the glycerol backbone of glycerolipids with saturated and monounsaturated fatty acids esterified mainly at the *sn*-1 and *sn*-2 position, respectively. Lowering the growth temperature led to a decrease in chain length of saturated fatty acids at *sn*-1 of all lipid classes and, at the same time, to an increased desaturation of 16:0 to 16:1 at sn-2 of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) (Sato *et al.*, 1979). In contrast in *A. variabilis*, which contained polyunsaturated fatty acids of 18 and 16 carbon atoms, localized respectively at *sn*-1 and *sn*-2 positions, growth at lower temperature indicated that only desaturation of C-18 acids was dependent on the growth temperature in all four major lipid classes (MGDG, DGDG, PG, and SL) (Sato *et al.*, 1979).

1.6 Effects of Membrane Lipid Unsaturation on Cyanobacterial Physiology and Biochemistry.

The glycerolipids are major components of bilayer membranes and provide the necessary enviroment for the functioning of membrane proteins (Doyle and Yu, 1985). The physical properties of glycerolipids and the membranes depends on the degree of unsaturation of the fatty acids that are esterified to the glycerol backbone of the lipids, and the molecular motion of these glycerolipids is affected by alterations in the extent of unsaturation of fatty acids (Chapman, 1975; Silvius, 1982).

Therefore, changes in the unsaturation of fatty acids should affect various functions of membrane-bond proteins, such as the photochemical and electron-transport reactions in thylakoid membranes, and the import and export of metabolites and protein across the plasma membrane (Murata and Wada, 1995). Low temperature, however, modifies the protein composition of the membranes as well as the carbohydrate status of the cells, thus making it difficult to establish whether a change in fatty acid unsaturation is of primary importance in the acclimation of the cell to low temperatures. Photosystem II (PSII) is embedded in the lipid bilayer of the thylakoid membrane, similar to other multiprotein complexes functioning in photosynthetic electron transport, although the overall role of glycerolipids and the effect of the degree of unsaturation in regulative associations are far from clear.

A mechanism can be proposed for low temperature-induced irreversible phenomena in *A. nidulans*. The plasma membrane and thylakoid membranes are barriers for ions and small molecules, whereas the outer membrane of the cell envelope is permeable to them. At the normal growth temperature, the plasma membrane and thylakoid membranes are both in the liquid crystalline state and are

impermeable to ions and small molecules. With a decrease in temperature, the thylakoid membranes go into the phase separation state and become permeable and ions and small molecules in the cytoplasm leak out of the cell, and those in the surrounding medium leak in. Under these conditions, physiological activities such as photosynthesis and photosynthetic ATP formation are reversibly diminished as revealed by a break in the Arrhenius plot (Murata *et al.*, 1975, 1983; Ono and Murata, 1979). This diminishes cellular metabolism leading to the death of the cell. Even when the temperature is raised to that which supports growth in unchilled cells, the concentrations of ions and small molecules are not recovered, thus resulting in irreversible damage of all the physiological activities of the cells.

The suggestion that the irreversible damage at low temperature in *A. nidulans* is induced by a phase transition in the plasma membrane, but not in the thylakoid membrane, has been verified by Vigh and his colleagues who selectively hydrogenated most of the unsaturated fatty acids in the plasma membrane, but not those of the thylakoid membrane in cells of *A. nidulans*. They observed parallel shifts in the degree of fatty acid saturation, the temperature for the onset of phase separation in the plasma membrane, and the temperature critical for K^+ leakage and the irreversible decline of photosynthesis (Vigh *et al.*, 1985). Gombos and Vigh (1986), using nitrate-starved cells of *A. nidulans* which contain an intact plasma membrane but degraded thylakoid membranes, also observed that the onset of K^+ leakage occurs at the same temperature which induces the onset in phase separation in the plasma membrane.

In *A. variabilis* which is tolerant of low temperature, no irreversible damage to photosynthesis nor electrolyte leakage occurs at 0°C (Murata *et al.*, 1984). These results are related to the finding that the plasma membrane is in the liquid crystalline

state above 0°C in this cyanobacterium (Ono and Murata, 1982; Wada *et al.*, 1984). In contrast, the thylakoid membranes enter the phase separation state above 0°C, and a clear break in the Arrhenius plot of photosynthesis appears (Wada *et al.*, 1984; Murata *et al.*, 1984). Therefore, in *A. variabilis* as in *A. nidulans*, phase transition of the thylakoid membranes does not induce irreversible damage in healthy cells, which is consistent with the mechanism for low temperature stress proposed for *A. nidulans*: that is, a phase transition in the plasma membrane is directly related to low-temperature damage.

The recent construction of plant and cyanobacterial constructs with specific changes in membrane lipid composition and in the level of fatty acid unsaturation has offered new possibilities for the direct study of the physiological importance of these membrane properties with respect to chilling tolerance and low temperature induced photoinhibition (Murata and Wada, 1995; Somerville, 1995). Several earlier studies (Wada *et al.*, 1990; Gombos *et al.*, 1992, 1994) succeeded in demonstrating increased susceptibility of transformed cyanobacterial cells to low temperature-induced photoinhibition when the unsaturation level of membrane lipids was reduced by genetic manipulation of fatty acid desaturase genes (Sippola *et al.*, 1998).

1.7 Ecophysiology of *Aphanizomenon* in The Baltic Sea.

The Baltic Sea is an enclosed, shallow sea (average depth 60m) with a surface area of 370,000 km^2 (Leppanen *et al.*,1988). It is subdivided into a number of areas: the Gulf of Bothnia, the Gulf of Finland, the Gulf of Riga, the Baltic proper, the Gotland Sea, the middle Bank, the Bornholm Sea and the Kiel Bight (Fig. 1.16). It is connected via narrow and shallow channels to the Kallegat and Skagerat that lead to the North Sea, and tides are virtually absent. Salinity in the Baltic Sea ranges

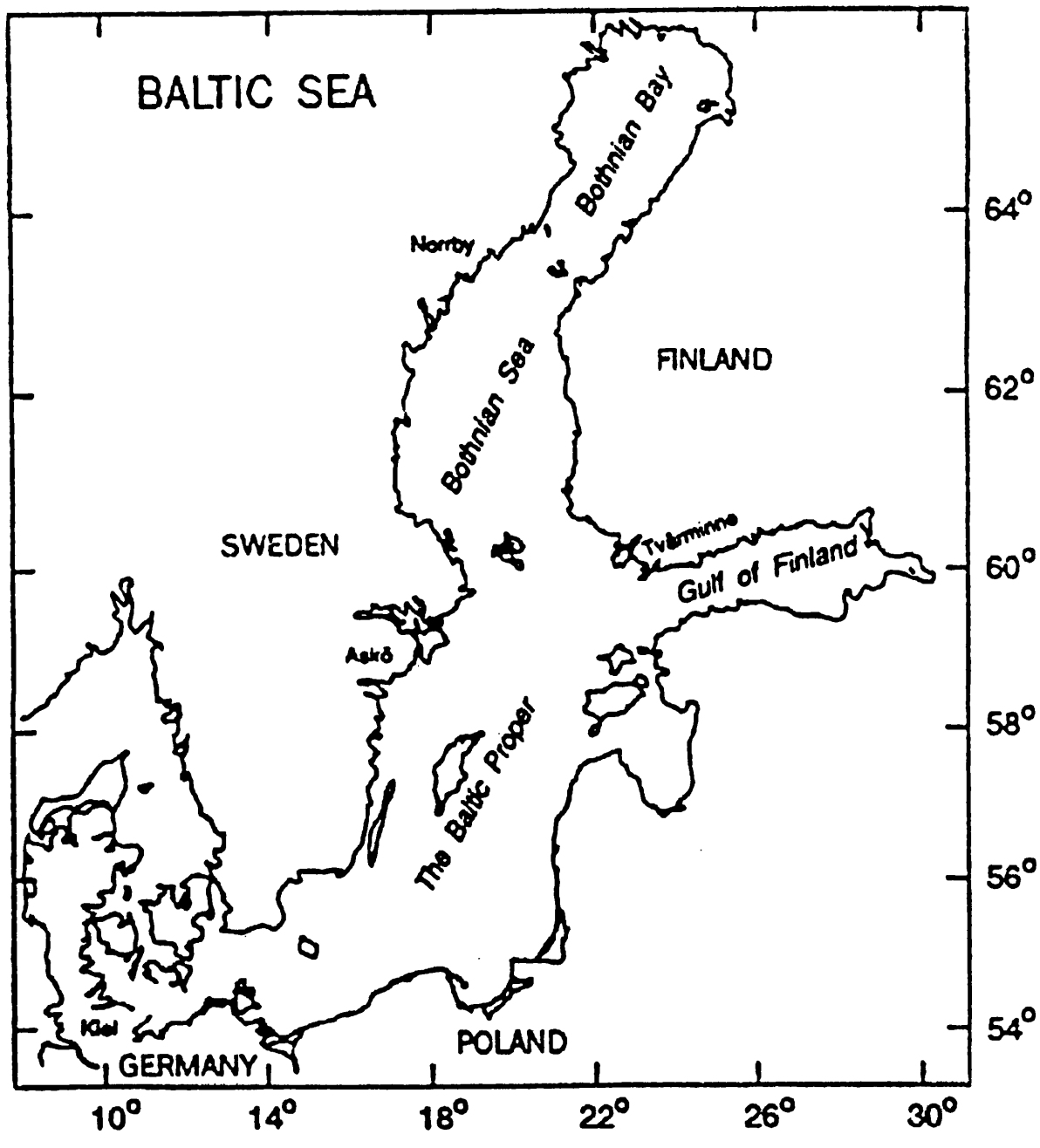


Fig 1.16 : Map of the Baltic sea taken from Kuparinen and Kuosa (1993).

from almost freshwater (1-4 practical salinity units; psu) in the eastern (Kahru *et al.*, 1994) and northern parts, to 7-8 psu in the south.

Summer Baltic sea blooms of cyanobacteria consisting of filamentous, nitrogen-fixing species of *Aphanizomenon* and *Nodularia spp.* are regular phenomena (Walsby *et al.*, 1995; Wasmund, 1997) characterized by several features fundamentally different from the phytoplankton vernal blooms. The bloom begins at the time of strongest seasonal thermal stratification (Kononen and Nommann, 1992). These bloom-forming cyanobacteria have gas vesicles that allow them to move vertically in the water column and to form colonies or aggregates that float to the water surface where they accumulate as dense scums (Walsby *et al.*, 1997; 1995). Despite the fact that these cyanobacterial blooms occur locally and for short periods of times (sometimes only weeks), estimates suggest that they fix between 10,000 and 130,000 tonnes of nitrogen per year (Larsson *et al.*, 1985), equivalent to 15-25% of the total nitrogen input into the Baltic Sea (Leppanen *et al.*, 1988). The position of the cyanobacteria in the water column is controlled by two mechanisms (Reynolds and Walsby, 1975). The first relies on the relatively steady rate of synthesis of gas vesicle production when compared to the growth rate. Thus as the cyanobacterium falls in the water column and the light becomes suboptimal, the rate of synthesis of gas vesicles is decreased, and the cells become less buoyant. In the second mechanism an increase in photosynthesis, and therefore in the concentration of osmotically active molecules of photosynthesis, results in an increase in turgor pressure, causing the collapse of weaker gas vesicles leading to the loss of buoyancy. The cyanobacterial cell will sink until photosynthesis, and the reduction in osmotically active molecules, reach a level where the decrease in turgor pressure results in an increase in gas vesicles and an upward movement of the cyanobacteria.

However, the conditions near the surface of the water are very unfavorable for the survival of vegetative cyanobacterial material, and cyanobacteria frequently undergo photooxidative damage after bloom formation (Abeliovich and Shilo, 1972; Boyd *et al.*, 1975). Filamentous cyanobacteria such as *Aphanizomenon flos-aquae* which are buoyant due the presence of gas vesicles (Walsby *et al.*, 1995) float near the water surface of the Baltic Sea and form blooms where they experience optimum light conditions. However, as a result of turbulence in the water column, they may be mixed to greater depths and as a result experience a decrease in temperature from about 25° at the surface to some 10°-15° at the thermocline (Fig 1.17) between the warm upper layer and the colder lower layer (Jones, 1999).

1.8 Aim of the Study and Programe of Work

Marine *Aphanizomenon sp.* are major contributors to the N_2 cycle in the Baltic ecosystem (Evans *et al.*, 2000). They are also major contributors to cyanobacterial blooms which are a nuisance and toxic or potentially toxic to man and livestock (see Kononen and Nộmmann, 1992). In the Baltic, *Aphanizomenon* sp. and other cyanobacteria are exposed during the summer to a wide range of temperature from a maximum of 30° whilst floating at the surface under calm conditions, to a minimum of 10° when they sink to the thermocline following a wind induced mixing event. The processes of sinking and returning to the surface during calm periods typically takes place over a relatively short time period, the mean velocity of *Aphanizomenon* in the Baltic has been determined as 22m day^{-1} (Walsby *et al.*, 1995). These organisms also need to adapt to longer term temperature changes as the temperature falls to its typical late summer value for the upper layer of

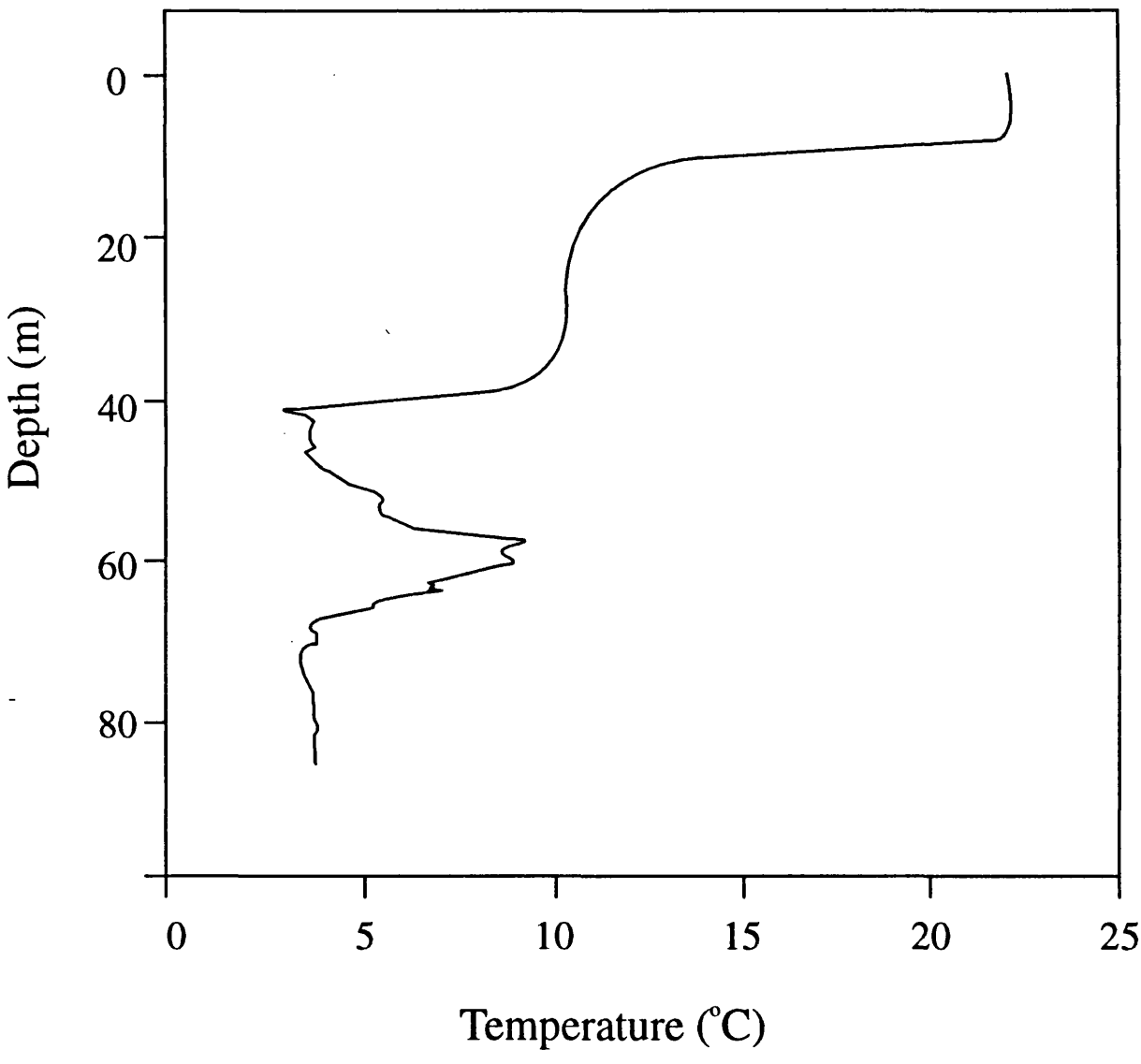


Fig 1.17 : Station 857 (10/8/94) Location: Off Bornholm Island (Jones, 1999).

17° – 18° (Kononen and Nômmann 1992) and to much lower temperatures during the winter.

To further elucidate the nature and significance of changes in membrane lipid fatty acid composition in temperature adaptation in natural populations of cyanobacteria in the Baltic Sea, the response of a unicyanobacterial strain of *Aphanizomenon* originally isolated from the Baltic, in terms of modulation of its membrane lipid fatty acid composition, to growth temperature and to temperature transitions within the normal physiological range experienced in its natural environment will be investigated.

In the initial phase of the programme of work the growth characteristics and fatty acid composition of *Aphanizomenon sp.* cultures grown isothermally at 28°, representative of the summer surface temperature of the Baltic, and at 15°, representative of the temperature at the base of the water column of the thermocline, will be established using chlorophyll and carotenoid as biomass markers together with cell dry weight. The changes in fatty acid composition in the total lipid fraction and major glycerolipid classes following (i) a downward temperature transition from 28° to 15°, and (ii) an upward temperature shift from 15° to 28° will then be determined. The stereospecific distribution of fatty acids in each individual glycerolipid class from *Aphanizomenon sp.* cultures grown at 28° will be established using lipase hydrolysis, which specifically hydrolyses the *sn*-1 ester position of glycerolipids; subsequently this approach will be used to further investigate the mechanism of the adaptive changes in fatty acid composition, through determination of the changes in fatty acid composition brought about at the *sn*-1 and *sn*-2 positions of each glycerolipid class by a downward temperature transition from 28° to 15°. To further investigate the metabolism of the glycerolipids and their constituent fatty

acids, radioisotope labelling studies using [1-^{14}C]-acetic acid and [1-(3)-^{3}H]-glycerol will be employed. The kinetics of labelling from each substrate will initially be established, and then *Aphanizomenon* sp. cells will be pre-labelled at 28° from ^{14}C-acetate and the labelling pattern of the glycerolipid classes and the total lipid fatty acid following a temperature down shift will be determined. Selected glycerolipid classes will be subject to further analysis by fractionation into individual molecular species by argentimetric-TLC. Dual radioisotope labelling studies, using a combination of [1-^{14}C]-acetic acid and [1-(3)-^{3}H]-glycerol will be used to further investigate metabolism of the glycerolipid fraction, in particular the biogenetic relationship of saturated and unsaturated glycerolipid molecular species during temperature adaptation.

The study described above will:

a) represent the first detailed study of lipid metabolism in a marine cyanobacterium.

b) establish the adaptive response of an ecologically significant cyanobacterial species and contribute to understanding of the ecophysiology of cyanobacterial blooms in the Baltic.

c) establish whether the stereospecific distribution of fatty acids within the glycerolipid classes of the marine cyanobacterium conforms to that generally accepted for cyanobacteria when classified by their fatty acid composition. (Murata *et al.*, 1992)

d) investigate the metabolism of glycerolipids during temperature adaptation, and in particular the time course and the formation of unsaturated fatty acids induced by a temperature-downshift.

e) evaluate the use of a radioisotope dual-labelling approach to investigate biogenetic relationships between molecular species within glycerolipid classes.

CHAPTER TWO

MATERIAL & METHODS

Chemicals, equipment, microorganisms and methods which were routinely employed in this present project are described in this Chapter. Chemicals were normally of the highest purity commercially obtainable.

2.1 Chemical Sources

Lipid standards including: monogalactosyldiglyceride, L-α- phosphatidyl-DL- glycerol, α-phosphatidyl-DG-glycerol, distearoyl, digalactosyldiglyceride , the methyl ester of 14:0, 16:0, 16:1, 17:0, 18:0, 18:1(9), 18:2 (9,12) and 18:3 (9,12,15), lipase enzyme (EC 3.1.1.3) type XI from *Rhizopus arrhizus,* primulin and molybdenum blue spray reagent for thin layer chromatography were obtained from Sigma Chemical Company (Poole, Dorest, U. K.). All other chemicals were of the highest purity available, and together with analytical grade organic solvents were obtained either from BDH Chemicals, Sigma U. K., or Aldrich Chemical Co. Acetic acid-1-^{14}C (specific radioactivity 7.13mCi / mmol [263.8 MBq/mmol]) was purchased from Sigma Chemical Company and converted to its sodium salt before use, and 1(3)-^{3}H-glycerol in ethanol solution (specific activity 130GBq/mmol, 3.50Ci/mole) was obtained from Amersham Nycomed plc (Amersham Nycomed, Buckinghamshire, U.K.). Ready safe™ liquid scintillation cocktail, containing phenylxylylethane surfactant was obtained from Beckman Instruments, Inc, U.S.A and plastic scintillation vials were obtained from L.I.P. Equipment and Services Ltd (Shipley, England). Water used for the preparation of liquid culture media and in other procedures was double distilled from glass. Thin layer chromatography plates (K6 Silica gel 60A with or without fluorescent indicator, 20×20cm, layer thickness 250μm) were purchased from Whatman International Ltd. (Maidstone, England). A 25m x 0.32mm I.D. BPX70 column (film thickness 0.25μm) was obtained from SGE

Analytical Products via Al-Gusabi Company in Jeddah. Gases for glc (He, H_2, air) and oxygen-free nitrogen (OFN) were of the highest purity (99.9%) and were purchased from Abdullah Hashem Company (Jeddah, Saudi Arabia).

2.2 Equipment

The following items of equipment were routinely used in this project (i) an International Equipment Company B-22 programable refrigerated centrifuge, (ii) a Shimadzu UV-1201 scanning UV-visible spectrophotometer, (iii) a console biological safety cabinet, (Forma Scientific class Π model 1100), (iv) a model KT-40 autoclave (ALP Co., Ltd) with drying process, (v) a Gallenkamp 101400.2.b illuminated, refrigerated orbital incubator (vi) a Bibby RE100 rotary evaporator, with water bath (vii) a Perkin Elmer _ 8700 gas liquid chromatograph with a flame ionization detector, equipped with a Bpx – 70 polar column, 0.25μm film and 25m x 0.22mm, I.D., (viii) a refrigerated, static incubator with dark and light cycle (Gallenkamp, model ICI180.WT2.C) (ix) a multi-purpose liquid scintillation counter (Beckman LS 6500) equipped with quench correction- and dual label programmes, (x) a Hanau fluotest ultra violet fluorescence inspection cabinet. (xii) a Mettler Toledo B154 semi-micro balance.

2.3 Safety Procedures

Working in a biochemical laboratory is potentially hazardous, so appropriate safety procedures were followed, especially when radioisotopes were in use. A laboratory coat, safety glasses and gloves were worn at all times in the laboratory, and a protective mask and fume hoods were used when handling volatile solvents and acids. The work with radioisotopes was confined to a small area in the

laboratory and all the waste radioactive product collected in a special container and removed by a specialist in radioactive waste disposal. Eating, drinking and mouth pipetting was prohibited in the laboratory, and all experiments were conducted in accordance with King Fahad Medical Research Center safety regulations.

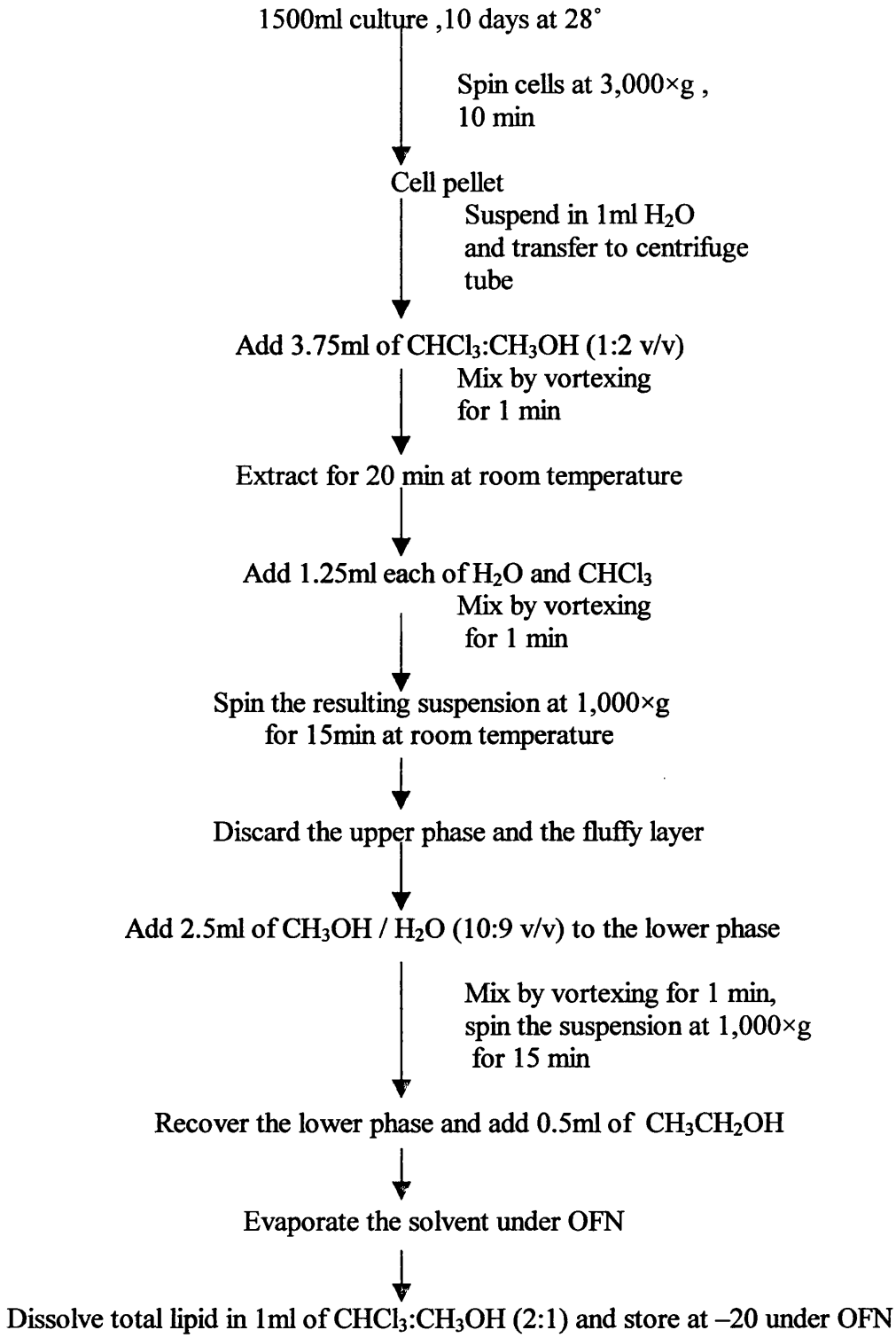
2.4 Microorganism and Culture Conditions

A culture derived from a strain of *Aphanizomenon* originally isolated from the Baltic Sea by Dr. K. Sivonen, Division of Microbiology, University of Helsinki, Finland was obtained from the Biochemistry Research Group, University of Wales Swansea, henceforth referred to as *Aphanizomenon sp.*, and was grown photoautotrophically in modified ASM-1 liquid medium lacking a fixed nitrogen source (Appendix 1) . The medium ingredients were mixed thoroughly and the pH value of the medium was adjusted to 7.6 by adding of a small volume of 0.1M HCl. Volumes of the medium were transferred into conical flasks, typically to occupy 1/2-3/4 of the flask volume, which were then loosely plugged with non- adsorbent cotton wool and sterilized in an autoclave at 121°, at a pressure of 1.5kgf/cm^2 for 15 minutes. When the flask attained room temperature, it was inoculated. Normally, 10% by volume of inoculum was used from a culture which had previously been grown for 10-12 days. Inoculations were carried out by sterile transfer in a console safety cabinet. Inoculated flasks were placed in an illuminated refrigerated orbital incubator on a 12h light (90μEm^{-2}s^{-1})/12h dark cycle; the speed of shaking was 120rpm, and normally cultures were maintained at a growth temperature of 28°.

2.5 Extraction of Total Lipid

Total lipid was extracted from *Aphanizomenon sp.* cells which had been harvested by centrifugation, normally at 3,000×g essentially according to the method of Bligh and Dyer (1959) as modified by Murata and Sato (1981), as described below.

After centrifugation of 1.5l of culture medium, the pellet of cyanobacterial cells was resuspended in 1 ml of distilled water. The suspension was transferred to a plastic centrifuge tube, and 3.75 ml of $CHCl_3$: CH_3OH (1:2, v/v) were then added and the suspension mixed by vortexing for 1 min. After extracting for a further 20 min at room temperature, 1.25 ml of each H_2O and $CHCl_3$ were added to the mixture which was mixed by vortexing for 1 min. The resulting suspension was then centrifuged at 1,000×g for 15 min at room temperature. The clear upper phase and the intermediate fluffy layer were withdrawn with a pasteur pipette and discarded, and 2.5 ml of CH_3OH / H_2O (10:9 v/v) was added to the lower phase and mixed by vortexing for 1 min, and the mixture centrifuged as in the previous step. The lower phase was recovered and transferred to a conical test tube. 0.5ml of C_2H_5OH was added to assist the complete evaporation of water, and the test tube was placed in a water bath and the solvent evaporated under a stream of oxygen-free N_2 (OFN). The resulting total lipid was normally dissolved in 1.0 ml of $CHCl_3$ /CH_3OH (2:1, v/v) and stored at -20° under an atmosphere of OFN. Details of the extraction procedure are summarized in Scheme 2.1.



Scheme 2.1 Extraction of total lipid from *Aphanizomenon sp.*

2.6 Transesterification of Total Lipid Fractions and Isolated Glycerolipid Fractions.

Samples (100-200µl) of total lipid extracts and isolated glycerolipid fractions were evaporated to dryness under OFN in glass tubes with Teflon-lined screw caps and 2ml of 2% H_2SO_4 / anhydrous CH_3OH was added. The tube was capped and heated at 55° for 15h (Christie,1982). The tubes were allowed to cool to room temperature and 2 ml of petroleum ether was added and mixed by vortexing for 1 min. 2ml of distilled water were then added and the tube inverted; the upper phase was aspirated and retained. Another 2ml of petroleum ether were added and the procedure repeated, and the upper phases combined . The solvent was evaporated to dryness under a steam of OFN after addition of 0.5ml C_2H_5OH to assist in the evaporation of water. The sample was redissolved in 100 µl of petroleum ether and stored at -20° under an atmosphere of OFN (see Scheme 2.2).

2.7 Thin Layer Chromatography (TLC)

2.7.1 Preparative TLC of the Fatty Acid Methyl Ester (FAME) Fraction.

The methyl ester samples prepared by transesterification of total lipid fractions and glycerolipid classes were purified by preparative TLC on either 20cm ×20cm or 10cm×20cm Whatman 60A silica gel-G coated plates. The plates were typically loaded with 100µl of FAME solution and, at the edges of the chromatogram and separate from the sample, 10 µl of an authentic solution of fatty acid methyl ester standard (5mg/ml); a solvent system of diethylether : petroleum

Evaporate samples of total lipid or isolated glycerolipid

to dryness under (OFN) in glass tubes.

↓

Add 2mlof 2% H_2SO_4 / anhydrous CH_3OH

↓

Cap the tubes and heat at 55° for 15h

↓

Allow the tubes to cool at room temperature

↓

Add 2ml of petroleum ether and mix by vortexing for 1 min

↓

Add 2ml of H_2O and invert the tube and aspirate the upper phase

↓

Add another 2ml of petroleum ether, re-extract, and combine the upper phase

↓

Add 0.5 ml of C_2H_5OH, evaporate the solvent to dryness under (OFN)

↓

Redissolve the sample in 100µl of petroleum ether

↓

Store the sample at –20° under OFN

Scheme 2.2 Transesterification of total lipid and isolated glycerolipid fraction

ether (1:9, v/v) was used to develop the chromatogram. When the solvent reached a height of 18cm, chromatograms were removed and allowed to dry at room temperature in a fume cupboard . The plates were then sprayed with 0.05% (w/v) primulin in $(CH_3)_2CO/H_2O$ (4:1 v/v), the solvent allowed to dry in fume cupboared, and sample visualized by inspection of the chromatogram under UV light (340nm). The gel area in the sample corresponding to the fatty acid methyl ester standard was scraped from the chromatogram, and transfered to 15ml of diethyl ether. The suspension was allowed to stand for 25 min with occasional swirling to extract FAMEs, and then filtered on a small scintered glass funnel under vacuum. The gel was washed twice with 5ml of diethylether and the filtrates combined. The solvent was evaporated by rotary evaporation and 1.5ml of petroleum ether added to dissolve the residue; the solution was transferred to a small vial and the solvent evaporated under a stream of OFN. The methyl ester fraction was redissolved in 0.2ml of petroleum ether and stored at -20° under an atmosphere of OFN prior to GLC analysis .

The recovery of FAME from transesterfication and preparative TLC was estimated by adding a known amount of internal standard heptadecanoic acid to representative total lipid extract before transesterification, then processing as described above in Section 2.6 above. An apparent recovery of 73% was routinely obtained; the method of calculation is shown below.

An aliquot (160µl) of a standard solution of heptadecanoic acid (260µg/ml) containing sufficient 17:0 to yield 43.7µg of heptadecanoic acid methyl ester was added to a lipid extract, and the FAME prepared and recovered from the chromatogram as described above was dissolved in 200µl of petroleum ether and 5µl was injected into the GLC, (see section 2.8 below). If the recovery was 100% this

aliquot would contain 1.09μg of 17:0 methyl ester. Under the GLC operating conditions used, 1.09μg of authentic 17:0 methyl ester injected directly was found to give a chromatogram peak area of 222.06 mm^2, whereas the area of the 17:0 methyl ester peak obtained after transesterification and recovery was 148.97 mm^2

The recovery of 17:0 methyl ester was therefore $\frac{148.97 \times 1.09 \times 100}{222.06} = 73.12\%$

2.7.2 Thin Layer Chromatography of Total Lipid

2.7.2(a) Preparative TLC of Total Lipid Extracts

Total lipid extracts prepared as described earlier were applied as a short band to silicagel-G TLC plates together with authentic samples of MGDG, DGDG and PG. The chromatogram was developed in a solvent of $CHCl_3$:CH_3OH:CH_3COOH:H_2O (170:30:20:7 v/v/v/v). After development the chromatogram was allowed to dry in a fume cupboard and components were visualized either by spraying with 0.05%(w/v) primulin in $(CH_3)_2CO/H_2O$ (4:1 v/v), and visualizing lipid bands under uv light, or by iodine visualisation as described in Section 2.7.2(b) below, and the corresponding areas scraped directly into vials and transesterified.

In the study of the stereospecific distribution of the fatty acids in glycerolipid molecular species, the MGDG fraction isolated as described above was subjected to further preparative TLC in a solvent of $CHCl_3$:CH_3OH:CH_3COOH:H_2O (85:15:5:1.5 v/v/v/v).

2.7.2(b) Analytical TLC of Total Lipid Extracts

Analytical TLC was carried out using the same solvent systems as described in section 2.7.2a above. Lipid fractions were visualised by the following procedures:

i) **Iodine exposure**: The chromatogram containing the lipid samples and glycolipid standards was removed from the tank and dried in the hood. When necessary to recover lipid samples, the plate was covered with a piece of glass to leave the standard and about 1cm of the lipid sample uncovered, and the chromatogram placed in a chromatography tank containing iodine crystals. Lipids became visible as brown spots, and can be provisionally identified according to their relative position (Christie, 1982)

ii) **α-Naphthol- conc. sulphuric acid (visualization of glycolipid)**: 0.5g of α-naphthol was dissolved in methanol-water (1:1 v/v) and the developed chromatogram was sprayed until the surface became moist. The plate was dried in the fume cupboard, and sprayed lightly with 95% sulphuric acid, then was heated at 120°. Glycolipids appeared as purple-blue spots, and other complex lipids as yellow spots Fig 2.1(Christie, 1982). MGDG, DGDG and PG were identified by reference to authentic samples. SL was identified by its characteristic colour reaction with α-naphthol/conc. sulphuric acid (Rf 0.19), and the heterocyst glycolipid was identified by its colour reaction and characteristic Rf (0.40).

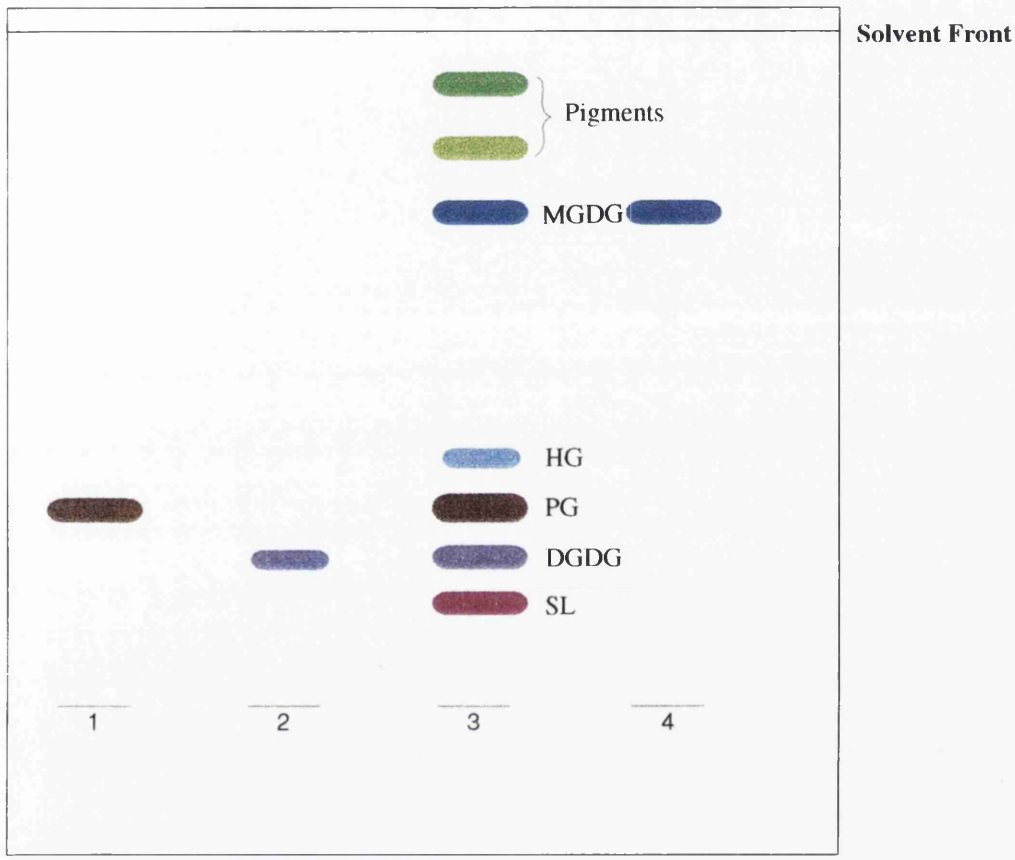


Fig 2.1 α - Naphthol : Conc. Sulphuric acid Visualisation of Silicagel - G total lipid chromatogram (Developing solvent : $CHCl_3$: MeOH : CH_3 COOH : H_2O 85: 15:5 :1.5 by vol.)

1- Distearoyl PG
2- Authentic digalactosyldiacylglycerol.
3- Aphanizomenon sp. total lipid extract.
4- Authentic monogalactosyl diacylglycerol

iii) **Molybdenum blue visualisation of phospholipids:** Chromatograms were sprayed lightly with molybdenum blue reagent (1.3% molybdenum oxide in 4.2M sulphuric acid). Phospholipids appear within 10 min. at room temperature as blue spots Fig 2.2 (Christie, 1982). A faint blue reaction was seen with this reagent in the area corresponding to the heterocyst glycolipid.

iv) **Sulphuric acid charring:** The chromatogram was sprayed until moist with 50% sulphuric acid and the plate was then heated at 180° until the lipids became visible as black deposits of carbon (Christie, 1982).

2.7.3.1 Silver Nitrate-Impregnated Silicagel-G Thin Layer Chromatography ($AgNO_3$-TLC)

2.7.3.2 Preparation of $AgNO_3$ –TLC Plates

A silicagel–G plate (20cm×20cm) was immersed in 5% (w/v) $AgNO_3$ in CH_3CN for 20 min in a clean pyrex dish . The plate was then removed and allowed to dry in a fume hood , and then activated at 105° for 30min and allowed to cool in reduced light before use.

2.7.4 Separation of Glycolipid Molecular Species and FAME by $AgNO_3$ –TLC

2.7.4 a Glycolipid molecular species

The MGDG fraction isolated as described earlier in Section 2.7.1 above was dissolved in 100µl of $CHCl_3/CH_3OH$ (2:1) and applied to a $AgNO_3$ – impregnated

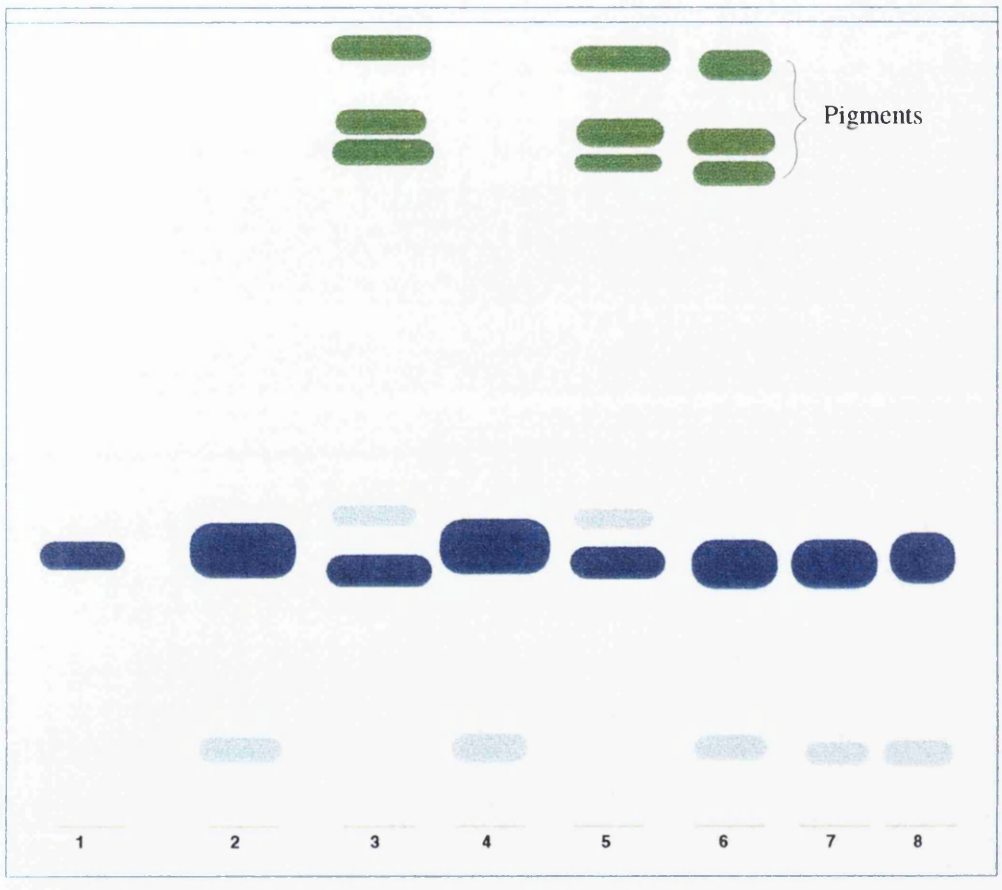


Fig 2.2 Molybdenum blue visualization of Silicagel-G total lipid chromaogram.
(Developing solvent : $CHCl_3$: CH_3OH : CH_3COOH : H_2O
85: 15: 5: 1.5 by vol.)

1= distearoyl PG
2= egg yolk PG
3= *Aphanizomenon* extract
4= distearoyl / egg PG
5= *Aphanizomenon* extract / distearoyl PG
6= *Aphanizomenon* extract / egg yolk PG
7= egg yolk PG 5µl
8= egg yolk PG 10µl

plate . The plate was developed in $CHCl_3$ / CH_3OH/H_2O (9:4:1 v/v/v) to a height of 19 cm . The chromatogram was removed and dried in fume hood then sprayed with 0.05%(w/v) primulin in $(CH_3)_2CO/H_2O$ (4:1 v/v) and the individual molecular species bands were detected by inspection under UV light. The fluorescent areas of gel corresponding to each molecular species were scraped out and transferred into centrifuge tubes containing 4ml of $CHCl_3$/ CH_3OH (2:1 v/v), and the suspension was vortexed and centrifuged at 1,000×g for 10 min to extract the molecular species from the gel. The supernatant was transferred to another centrifuge tube using a pasteur pipette and 1ml of H_2O was added and mixed by vortexing for 1min. The suspension was centrifuged at 1,000×g for 1min and the upper layer was removed and discarded. To remove any remaining traces of $AgNO_3$ the lower layer was then washed twice with 1ml of H_2O and centrifuged at 1,000×g for 10min. The lower layer was transferred to a dry test tube and the solvent evaporated under a stream of OFN. The resulting molecular species was transesterified (see section 2.6) and the fatty acids in each molecular species were identified by GLC as described in section 2.8.

2.7.4 b Separation of the FAME fraction from total lipid extracts

The FAME fraction isolated as described in Section 2.7.1 earlier was dissolved in 100µl of $CHCl_3$:CH_3OH (2:1v/v) and applied to a $AgNO_3$–TLC plate. After development in a solvent of diethylether:petroleum ether (3:17 v/v), the four FAME fractions were detected by UV inspection after spraying with primulin (0.05% w/v in $(CH_3)_2CO/H_2O$ 4:1 v/v), and the bands were recovered from the gel and processed as described in section 2.7.4 (a) above.

2.8 Gas Liquid Chromatography [GLC]

Fatty acid methyl esters were identified and quantified using a highly 'polar' capillary column (BPX70, 0.25μm film, 25m×0.22mm i.d). The chromatograph was equipped with a flame ionization detector. The initial oven temperature was 130°, and temperature programming began immediately on injection at a constant rate of 2° / min to the final temperature of 210°. Injections were made in the split injection mode (10:1) at an injector temperature of 160° using helium as carrier gas at a flow rate of 10ml/min. Normally 1-5μl of the solution of FAMEs in petroleum ether were injected into the GLC. Individual components were identified by comparison of their retention times with authentic fatty acid methyl ester standards, and by comparison with a GLC-MS total ion chromatogram determined under similar conditions at UWS (Figure 2.3a). A representitive chromatogram is shown in Fig 2.3b. The relative % composition of fatty acids was calculated from the area of the individual peaks of interest in the chromatogram data report. Absolute quantification of fatty acid methyl esters was obtained by injection of known amounts of an appropriate authentic FAME standard and determination of peak area. The amount of unknown FAME was then calculated by proportionation.

2.9 Determination of Biomass – Marker Pigments

Cyanobacterial culture (duplicate 5ml samples) was centrifuged at 3,000×g for 10 min, and the resultant pellet was resuspended in 2 ml ethanol and left for 15 h at room temperature in the dark.

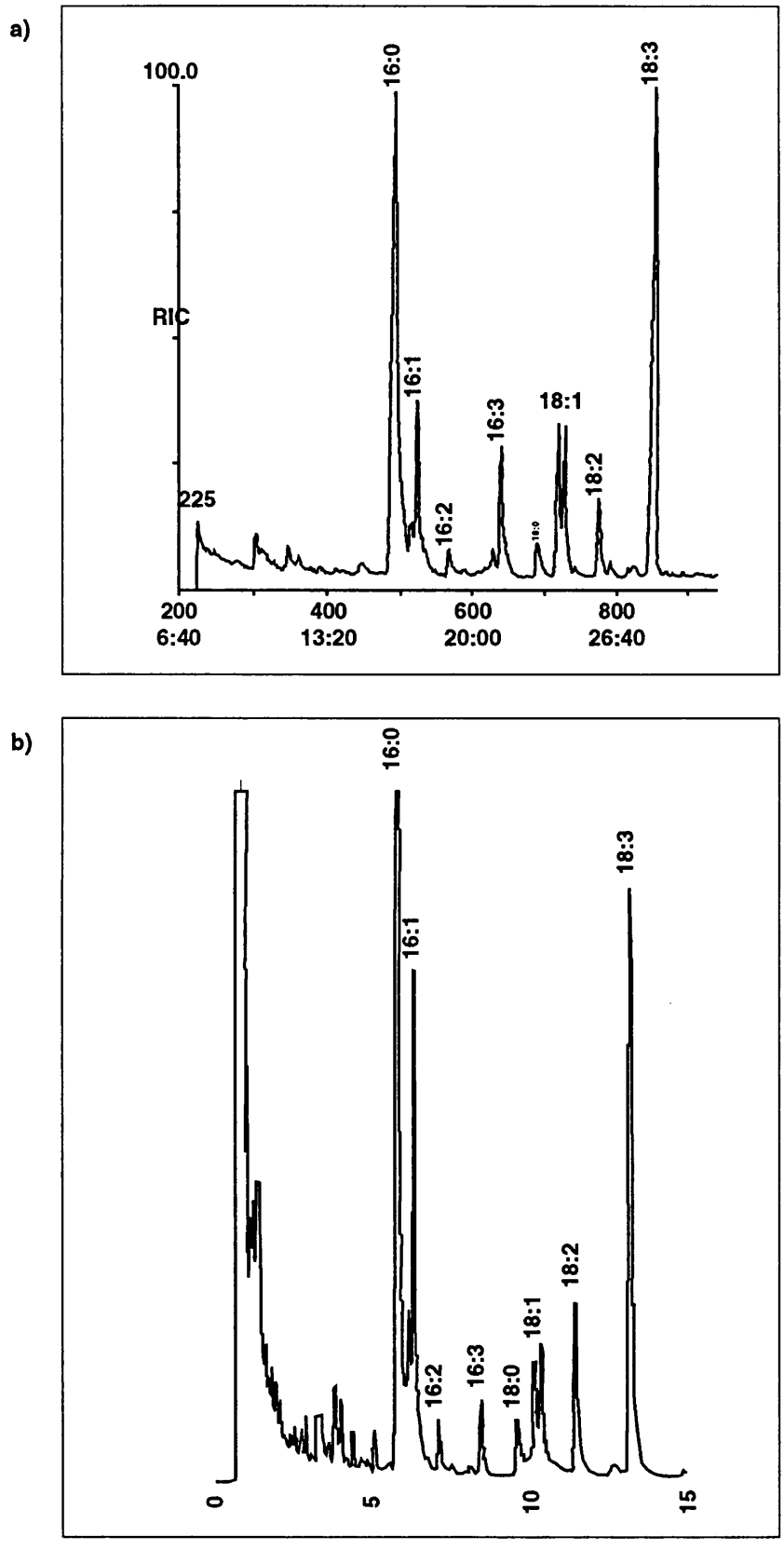


Fig 2.3 Gas Chromatography of *Aphanizomenon* sp fatty acid methyl esters ; a) mass chromatogram (T.J. Walton , Personal Communication) , b) gas liquid chromatogram (Flame ionisation detector)

2.9.1 Chlorophyll

The absorbance at 665nm of the resultant ethanolic solution was determined in a scanning spectrophotometer (Stal *et al.*, 1984), and given that under these conditions;

Absorbance at 665nm × 13.8 = chlorophyll a conc. (mg/L)

The chlorophyll conc.(mg/100ml culture) was calculated as follows:

$$\text{Chlorophyll concentration} = \frac{A_{665} \times 13.8 \times 2 \times 100}{5 \times 1000} \text{ (mg/100ml)}$$

2.9.2 Carotenoid

Carotenoid extracts from culture (5ml duplicates) was prepared as described above (Sec.2.9 (i)). The absorbance of the ethanolic solution was measured at 431 nm (Fork *et al* ., 1979) using an $A_{1cm}^{1\%}$ value of 2.5×10^3. The concentration of carotenoid (μg / 100ml culture) was obtained as follows:

$$\text{Carotenoid concentration (μg / 100ml culture)} = \frac{A_{431} \times 2 \times 10^6}{2.5 \times 10^3 \times 5}$$

2.10 Determination of Cyanobacterial Cell Dry Weight

Duplicate 10 ml samples of culture were taken and dried to constant weight in small pre-weighed glass beaker overnight at 105°. The beaker was removed from the oven and placed in a desiccator until it reached room temperature, weighed on a semi-microbalance and the dry weight of cells present in 100 ml calculated.

2.11 Temperature Adaptation Studies (28° to 15°)

Aphanizomenon sp. culture was grown in 1500ml of modified ASM-1 medium contained in a 2L conical flask for 10 days at 28° as described above . The incubator

temperature was then reduced to 15°. Under these conditions the temperature of cultures in the the incubator were found to have fallen to 15° within 1.5h of the incubator temperature being lowered. Samples of culture for determination of dry weight (10 ml), chlorophyll and carotenoid levels (5ml), and total lipid extraction (130ml) were taken immediately after decreasing the temperature, after a further 12h and then every 24h for the following 3 days.

2.12 Lipase Hydrolysis Procedure for Stereospecific Analysis of Cyanobacterial Glycerolipids

The distribution of fatty acids at the *sn*-1 and *sn*-2 positions of the individual glycerolipids was established by a procedure based on that described by Sato and Murata (1988), which was developed from the procedures described by Fischer *et al.*, (1973).

2.12.1 Enzymatic Hydrolysis of Glycerolipids with Lipase

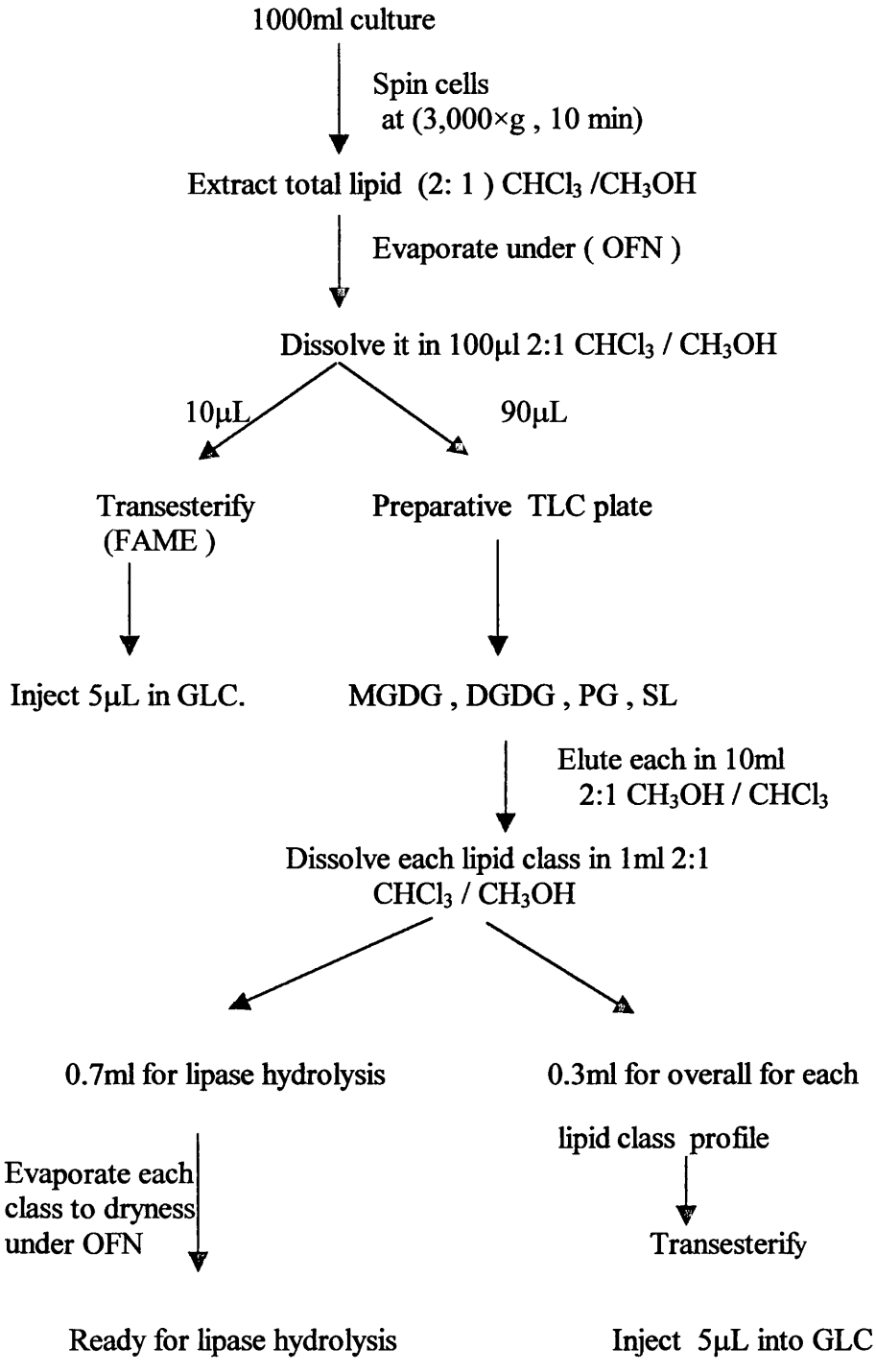
Each glycolipid fraction recovered from preparative silica gel-G TLC was dissolved in 1.0ml of $CHCl_3$:CH_3OH (2:1 v/v) and 0.7ml was taken and evaporated to dryness under OFN in a small sample bottle. The lipid was redissolved by vortexing in 0.9ml of 50mM Tris-HCl buffer (pH 7.2) containing 0.05% (w/v) Triton-X100. *Rhizopus* lipase (5µl containing about 2000 units in 10mM phosphate buffer, pH 6.0) was added and the mixture was incubated at room temperature with gentle shaking. Incubations were continued for the period appropriate to each glycerolipid class (see Table 2.1). The reaction was stopped by the addition of 3ml of CH_3CH_2OH, and the solvent then removed by rotary evaporation. A mixture of 100µl of $CHCl_3$: CH_3OH (2:1 v/v) was added to the residue and the mixture swirled

gently in an ice bath to dissolve . The remaining 0.3 ml of the original glycolipid sample (Scheme 2.3) was retained for transesterification as described previously (Section 2.6).

2.12.2 Multiple Development Thin Layer Chromatography of Lipase Products

2.12.3 Isolation of *sn*-1 Fatty Acid and Lysoglycerolipid.

The lipase hydrolysis products were separated by preparative thin layer chromatography on silica gel plates using successive development with two solvent systems. After the first development in petroleum ether : diethylether : formic acid (25:20:1 v/v/v) to about 1cm from the top, the chromatogram was dried in a fume cupboard and immediately sprayed with 0.05% primulin in $(CH_3)_2CO$ / H_2O (4:1v/v), then allowed to dry in fume cupboard and inspected under uv light. The band containing free fatty acid was scraped off, and the gel transferred to a transesterification tube and 2ml of $MeOH/H_2SO_4$ reagent was added. For subsequent isolation of the lyso-compound, the chromatogram was placed in a solvent system consisting of $CHCl_3$:CH_3OH:H_2O in the ratio appropriate to each lipid class (see Table 2.1). When the solvent front neared the discontinuity in the silica gel, the chromatogram was removed from the solvent, allowed to dry in a fume cupboard and and sprayed again with 0.05% primulin in $(CH_3)_2CO/H_2O$ (4:1 v/v) and inspected under uv light. The band containing the lysoglycerolipid product was scraped off, transferred to a transesterification tube and 2ml of $MeOH/H_2SO_4$ was added . The samples of the original glycerolipid and the lysoglycerolipid were transesterified by



Scheme 2.3 Lipase hydrolysis procedure for stereospecific analysis of cyanobacterial glycerolipids.

heating at 55° overnight, and the FAMEs isolated and analysed by GC as described earlier. The fatty acid composition at *sn*-2 was determined directly from the lysoglycerolipid samples, and the composition at *sn*-1 by difference between the original glycerolipid and the corresponding lysoglycerolipid, according to the following expression which is reported to give more reliable results than direct analysis of the *sn*-1 fatty acid yielded by lipase hydrolysis.

$$sn\text{-1 fatty acid} = \left[\begin{array}{l}2 \times \text{glycerolipid overall} \\ \text{fatty acid composition}\end{array}\right] - \left[\begin{array}{l}\text{glycerolipid } sn\text{-2 fatty} \\ \text{acid composition}\end{array}\right]$$

The protocol used for sample preparation for the stereospecific analysis of glycerolipids during temperature transition is shown in Scheme 2.4

2.13 Studies of Lipid Metabolism by Use of Radioactive Substrates

2.13.1 Radioactivity Determination by Liquid Scintillation Counting

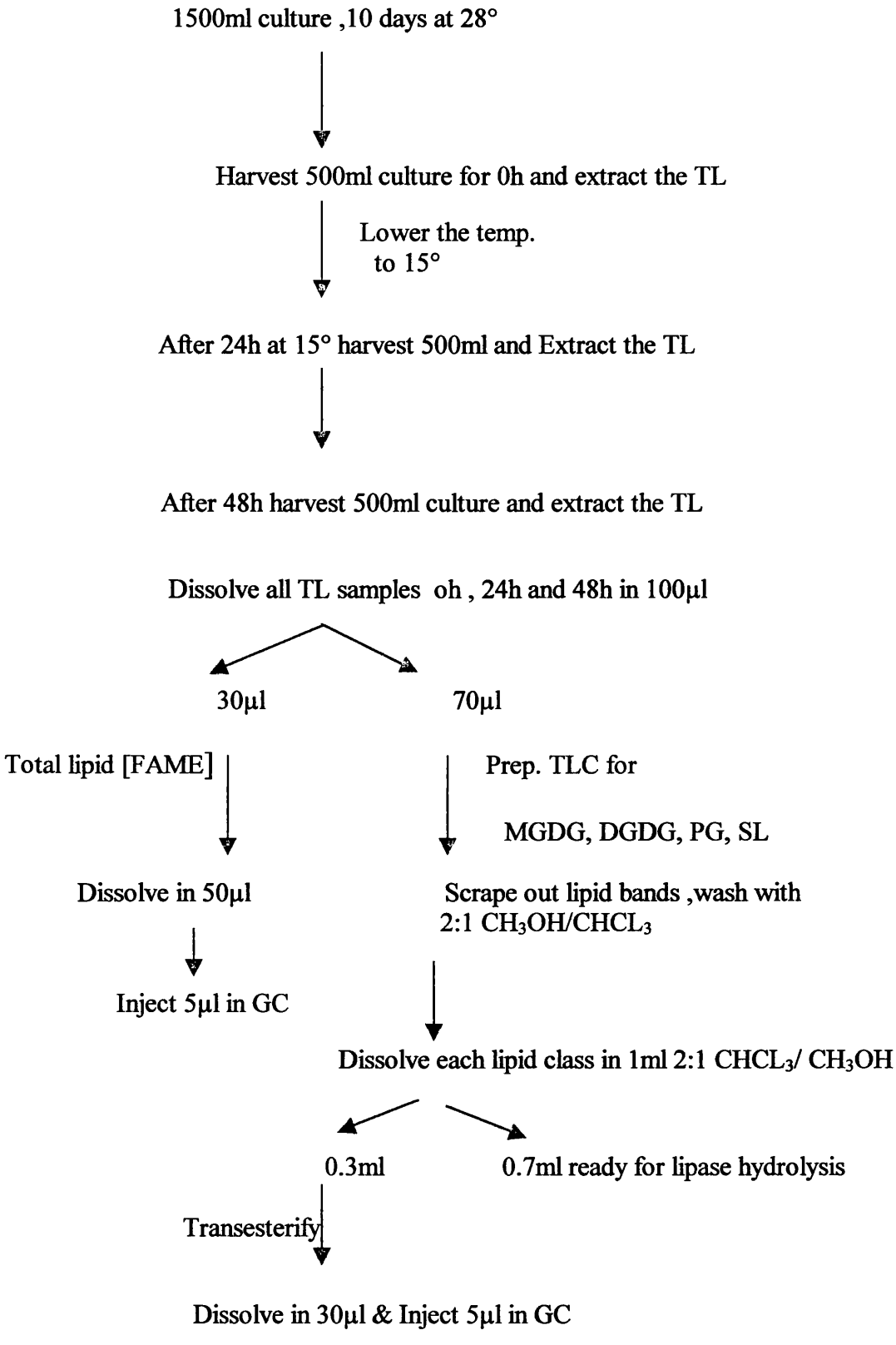
Radioactive samples were normally added to 5ml of scintillation cocktail contained in a 20ml plastic scintillation vial, and the samples were counted for 5 min in a Beckman LS 6500 scintillation counter using the appropriate dpm counting programme. The parameter group setting of each programme used is shown in Appendix 1. (^{14}C, ^{3}H and dual labelling ^{3}H+ ^{14}C). Samples containing $AgNO_3$ impregnated gel were counted within 3 min of transfer to scintillant and the counting time was minimized to 2 min to reduce the quench value (H#).

2.13.2 Incubations with [1- ^{14}C] – Acetic Acid

In studies of the kinetics of lipid labelling, typically 1500 ml of culture were grown at 28° in the shaking incubator under the normal lighting regime for 10

Table : 2.1 Lipase Hydrolysis Procedure

Glycerolipid	Incubation time/min	Solvent System for 2nd TLC Development $CHCl_3$: CH_3OH:H_2O (v : v : v)	Rf of the Lysoglycerolipid Product
MGDG	10	35 : 6.5 : 0.5	0.77
DGDG	25	35 : 12 : 0.5	0.41
PG	45	35 : 9 : 0.5	0.64
SL	50	35 : 11 : 0.5	0.27

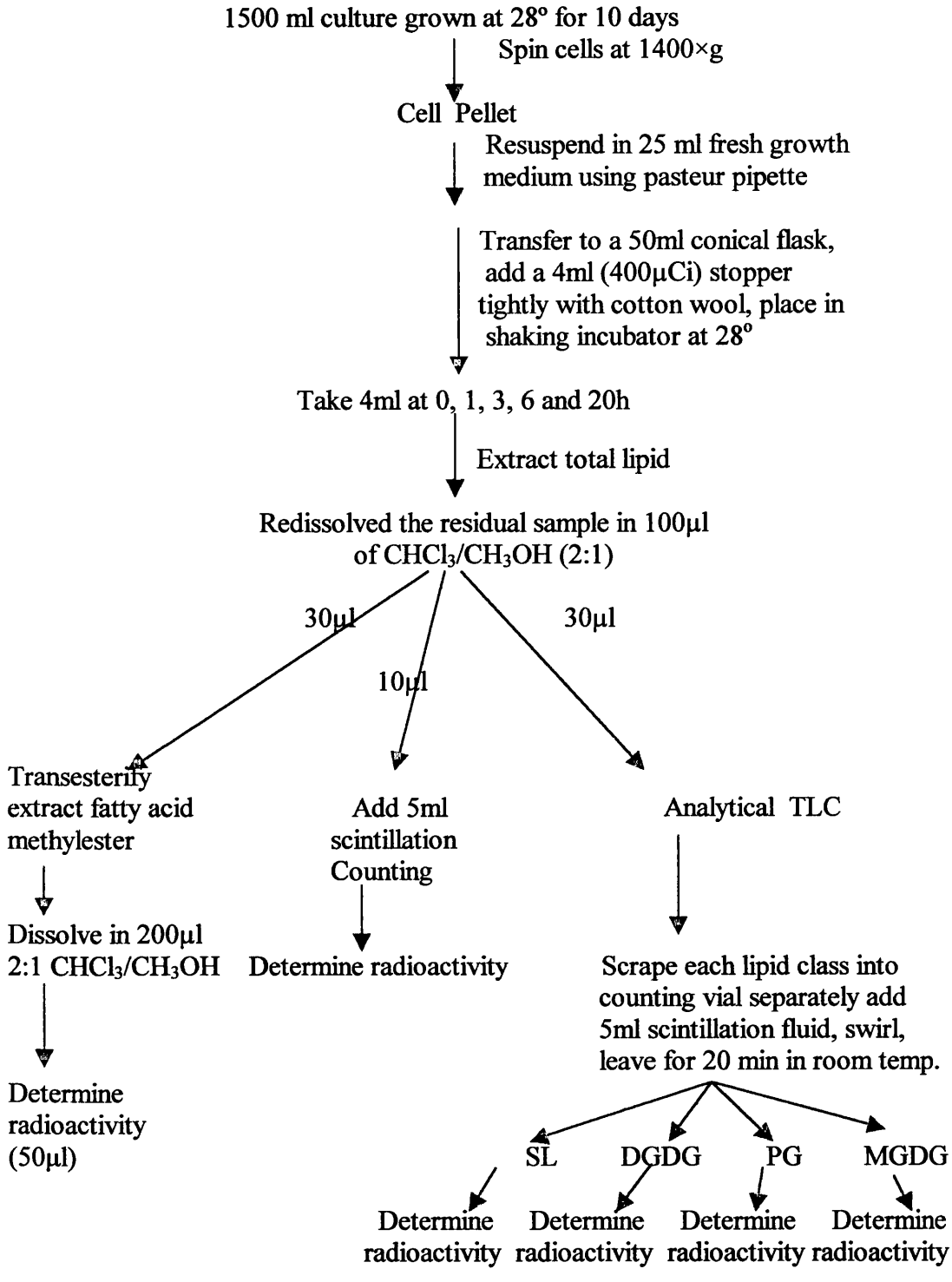


Scheme 2.4 Protocol for sample preparation for stereospecefic analysis of glycerolipids during temperature transition

days. At day 11 the *Aphanizomenon* cells were harvested and centrifuged at 1430×g at room temperature. The pellet was gently resuspended by means of a pasteur pipette in 25 ml of fresh medium and transferred to a 50ml conical flask. [1-^{14}C] – Acetic acid was converted to its sodium salt by addition of excess (1ml) 5mM aqueous sodium bicarbonate solution, and 40μCi was added to the *Aphanizomenon sp.* cells; a 4ml sample was harvested immediately (0 hour sample) and total lipid was extracted according to the method described earlier (Section 2.5). The remaining cell suspension was placed in the shaking incubator at 28°, and 4 ml of the labeled cells were harvested after 1, 3, 6, and 20 hour and the total lipid extracted. The total lipid samples were redissolved in 100μl $CHCl_3/CH_3OH$ (2:1 v/v) from which 10μl aliquots were transferred directly to scintillation counting vials, and counted (see section 2.13.1). Total lipid aliquots (30μl) were transesterified, and the resulting FAMEs fraction dissolved in 200μl $CHCl_3/CH_3OH$ (2:1 v/v), 50μl of which were counted. Two 15μl (duplicate) aliquots were applied to an activated TLC plate for the separation of individual lipid classes (Section 2.7.2a), then each lipid class was scraped separately and directly transferred to a counting vial containing scintillant. Details of the procedure are summarized in Scheme 2.5. The sample was swirled, left for 20 min at room temperature, then counted. The remaining total lipid sample (30μl) was used for separation of MGDG molecular species as described earlier in Section 2.7.4 a.

2.13.3 Incubations with [1(3)-^{3}H]-glycerol.

An essentially identical procedure to that described above for [^{14}C]-acetate labelling was followed except that 300μCi (130GBq) of [1(3)-^{3}H]-glycerol (200μl; specific radioactivity) was used.



Scheme 2.5 Protocol for incubation and lipid extraction from *Aphanizomenon* sp. cells used in labelling studies with sodium [1-^{14}C] –acetate.

2.13.4 Incubations with Sodium ^{14}C- Acetate to Study Lipid Metabolism During Temperature Adaptation.

Three culture flasks each containing 1500ml of medium were used for these experiments. Cultures were grown at 28° in the orbital incubator for 10-12 days, after which the cells were harvested by centrifugation at 1430 ×g for 10 min. The pellets were resuspended, combined and transferred to a 100ml conical flask containing 50ml fresh, sterile growth medium. Sodium [1-^{14}C]-acetate (400μCi; 0.6ml, prepared as described in Section 2.13.2 above) was added and the flask was stoppered lightly with cotton wool and placed in the illuminated orbital incubator in a secure, stable position. The membrane lipids were labelled by incubation of the cells for 3h, after which the cells were gently harvested by centrifugation at 1430×g at room temperature. The cells were washed by resuspension in 30ml of fresh growth medium, then the cells were centrifuged again at 1430×g for 10min to remove extra-cellular substrate. The supernatant was aspirated and the washing procedure repeated. The resulting pellet was resuspended in 50ml of fresh growth medium containing 10mM unlabelled sodium acetate and transferred to a 100ml conical flask. A 10ml sample was taken and the lipid extracted immediately (time '0' sample) and the flask was then replaced in the shaking incubator and the temperature setting lowered to 15°. Further 10ml samples were harvested after 12h, 24h and 48h and the total lipid was extracted from each sample. For each sample 10μl aliquots of total lipid were counted in duplicate, and duplicate 15μl aliquots were subjected to analytical silica gel-G TLC, to determine the radioactivity in the MGDG, PG, DGDG and SL fractons by scintillation counting, and 30μl aliquots were transesterified and the FAME isolated as described in (section 2.6) and analysed by $AgNO_3$ TLC. The four bands corresponding to saturated, monoenoic, dienoic and

trienoic fatty acid methyl esters were scraped directly to counting vials and their radioactivity determined by scintillation counting. The remaining total lipid sample (20µl) was subjected to preparative silica gel G chromatography and the band corresponding to MGDG was recovered and redissolved in 100µl of 2:1 (v/v) $CHCl_3$:CH_3OH. Duplicate 30µl samples from the recovered MGDG sample were applied to a $AgNO_3$ TLC plate, and each band seen following spraying with primulin and uv inspection was scraped directly to a counting vial and the radioactivity determined by scintillation counting.

A similar labelling protocol to that described above was used for the ^{3}H:^{14}C-double labelling studies during temperature transition, except that the radioactive substrate contained [1-^{14}C]-acetate and [1(3)-^{3}H]-glycerol (40µCi and 200µCi respectively; further details in Scheme 2.6). The dual label lipid samples were analysed according to the flow scheme shown in Scheme 2.7.

2× 1500ml culture grown at 28° for 10 days

↓ Spin cells at 1400 ×g, cell pellet

Resuspend in 50ml fresh sterile growth medium using pasteur pipette

↓

Transfer to a 100ml conical flask, add 40µl (400µCi) ^{14}C-acetic acid and 200µl (200µCi) ^{3}H-glycerol, stopper tightly with cotton wool, place in shaking illuminated incubator , incubate for 3h at 28°.

↓

Centrifuge at 1400 ×g at 28°, decant supernatant, resuspend in 25ml steril growth medium at 28°, repellet and decant supernatant (repeat suspension, decanting step)

↓

Resuspend cells in 30ml in sterile growth medium at 28° containing steril 0.6ml 10mM sodium acetate.

↓

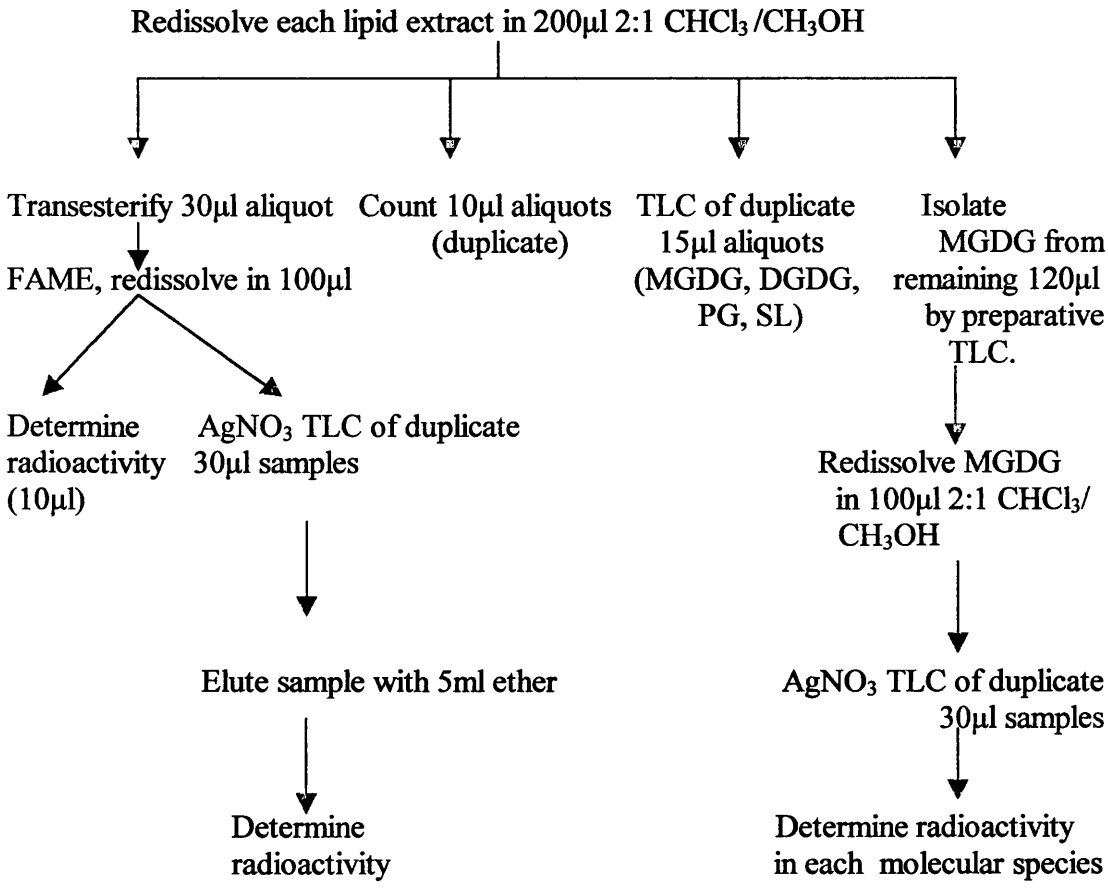
Take 10ml sample and extract lipid immediately (0.1h)

Transfer remaining sample back to the incubator at 15°

↓

Take 10ml samples after 12h, and 24h

Scheme 2.6 Protocol for the ^{3}H:^{14}C-double labelling procedure during temperature transition.



Scheme 2.7 Protocol for analysis of ^{3}H:^{14}C double labelled lipid extracts.

CHAPTER THREE

Growth Characteristics and Fatty Acid Composition of *Aphanizomenon* sp. laboratory cultures.

3.1 Introduction

Widely different rates of growth have been reported for different species of cyanobacteria in nature, which may be a reflection in part of their differing morphological and physical characteristics. Observed growth rates could be an adaptation to the natural diel cycle (24h cycle) and the cyanobacterium's predominantly photoautotrophic metabolism. For example a marine *Anabaena sp.* has a generation time of 4.3h (which corresponds to 5.6 generation per day) when grown under optimum conditions, whilst *Anacystis nidulans* has a doubling time of only 2 hours (12 generations per day) when grown under optimum conditions (Fay, 1983). The main factors to determine the growth rate of cyanobacteria are light, pH, nutrient concentrations and temperature. Temperature does not seem to be the most significant factor in determining growth rates of cyanobacteria (Fay, 1983) but is one of the most important environmental factors that influence the fatty acid composition of lipids of many organisms including cyanobacteria. (Hazel, and Posser, 1974; Kates and Hagen, 1964; Wada and. Murata, 1990). *Aphanizomenon sp.* in the Baltic sea will be exposed to a temperature range between about 25° at the surface to 15° at the thermocline, as discussed in chapter 1.2.

In this chapter the growth characteristics and fatty acid composition of *Aphanizomenon sp.* grown in laboratory culture for up to 25 days in a medium free of a fixed nitrogen source were studied under two different temperatures. The temperatures selected for the studies in this chapter were 28° (high temperature) corresponding to the temperature to which *Aphanizomenon* colonies floating at the surface of the Baltic would be exposed, and 15° (low temperature) corresponding to the temperature at the Baltic thermocline, to which cyanobacteria would be exposed

on sinking in the water column. The growth characteristics were investigated by determination of two markers for cyanobacterial biomass, chlorophyll a and carotenoid, which are present in the lipid bilayer of cyanobacterial membranes (Fay, 1983) as described in chapter 2.9. Growth of the cultures was also determined by measuring the dry weight of the cells contained in aliquots of culture media sampled over the growth period (Raymond *et al.,* 1964). The effect of growth temperature on the fatty acid composition of *Aphanizomenon sp.* was studied by extraction of the total lipid at 24h intervals during the growth period of cultures grown up to 25 days at (i) 28° and (ii) 15°, and analysis by GLC of the fatty acid methyl esters (FAMEs) as described in chapter 2.6

3.2 Results of Studies on the Growth Characteristics of *Aphanizomenon sp.* Cultured at 28°

3.2.a Growth Curve Determination Based on Chlorophyll Content of Cultures.

Growth in *Aphanizomenon sp.* cultures was measured by determining the concentration of chlorophyll a over a period of up to 25 days (0h-600h) following inoculation. Duplicate 5ml samples of *Aphanizomenon* culture were taken and the chlorophyll a content determined as described in chapter 2.9.1 above. The resulting growth curve (Fig 3.1) showed that the concentration of chlorophyll a gradually increased in the first 168h following inoculation, but after that period there was a sharp increase in chlorophyll a concentration, which continued to increase for the period between (168h – 240h), after which the concentration declined slightly

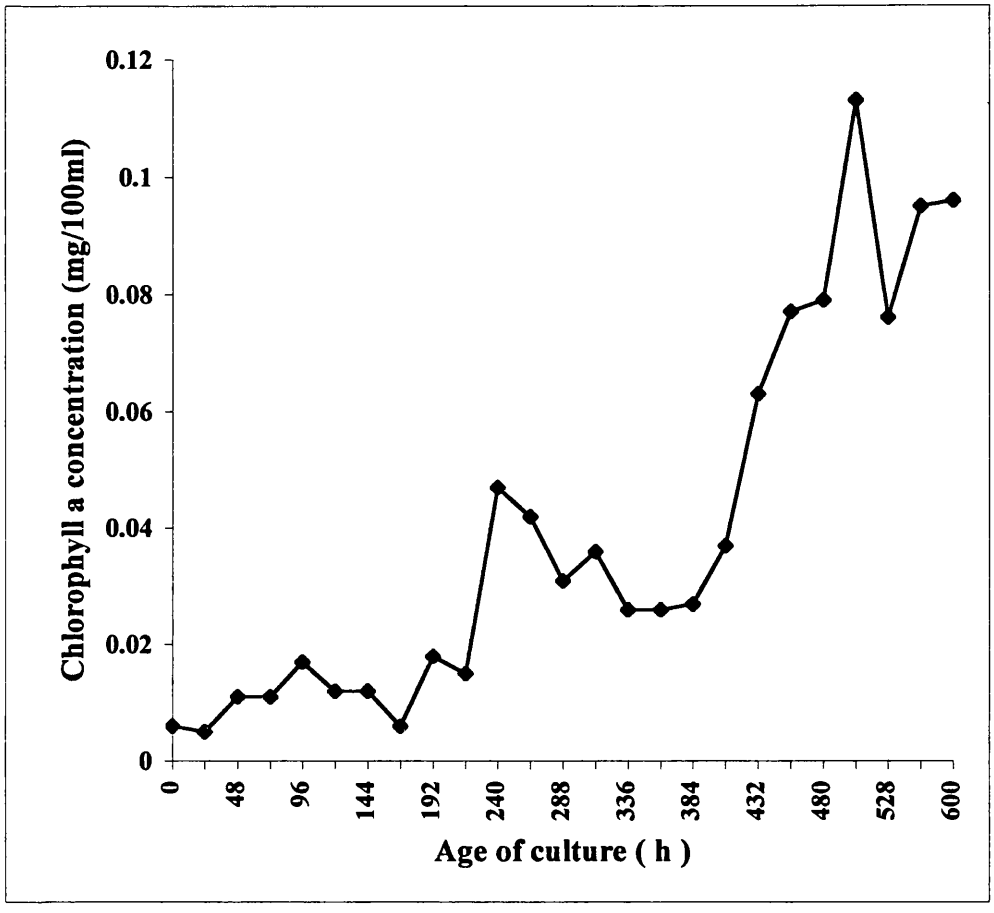


Figure 3.1 Concentration of chlorophyll a (mg / 100ml) in *Aphanizomenon* sp culture grown at 28° for 25 days

until 360h and then started to increase again. This suggested that there may be four phases in the growth of *Aphanizomenon sp.* cultures, composed of a lag period from (0h – 160h), an exponential phase from (168h – 264h) following by a second lag period (288h-384h) and then a further exponential phase from (408h – 552h).

In view of the unexpected form of the growth curve the experiment was repeated under the same growth conditions (Fig 3.2). A similar pattern in the growth curve was observed with a lag period between 0 – 120h, followed by an increase in chlorophyll concentration between 120 – 264 hours followed by a further lag period (216h- 264h) and then a sharp increase between 264 – 312h.

3.2.b Growth Curve Determination Based on Carotenoid Content of cultures.

The carotenoid content of 5 ml samples of *Aphanizomenon sp* culture was determined as described in chapter 2.9.2 for a 25 day culture period. The resulting growth curve (Fig 3.3) indicated that there was no significant increase in the carotenoid level for the first 168h, after which (168h – 264h) there was a substantial increase in the carotenoid concentration followed by a short plateau after which the level of carotenoid increased again between 408h to the end of the growth period, and supported the apparent presence of four different phases of growth in the *Aphanizomenon sp* cultures indicated by the chlorophyll a levels (Fig 3.1). When the experiment was repeated over 14 day growth period (0h-336h) under the same conditions, a similar growth pattern was observed (Fig 3.4) and with a lag period of 96h followed by an exponential phase between 96h – 288h, a second lag phase and a

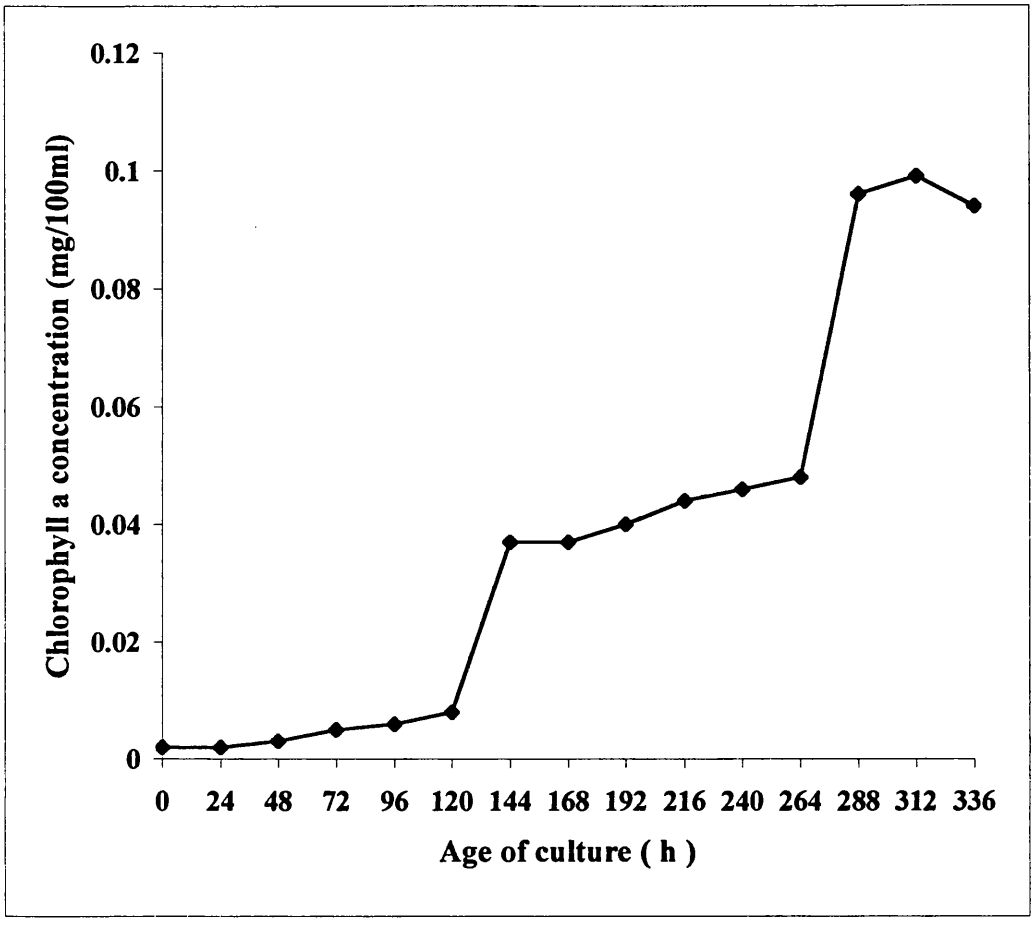


Figure 3.2 Concentration of chlorophyll a (mg / 100 ml) from *Aphanizomenon* sp. culture grown at 28° for 14 days.

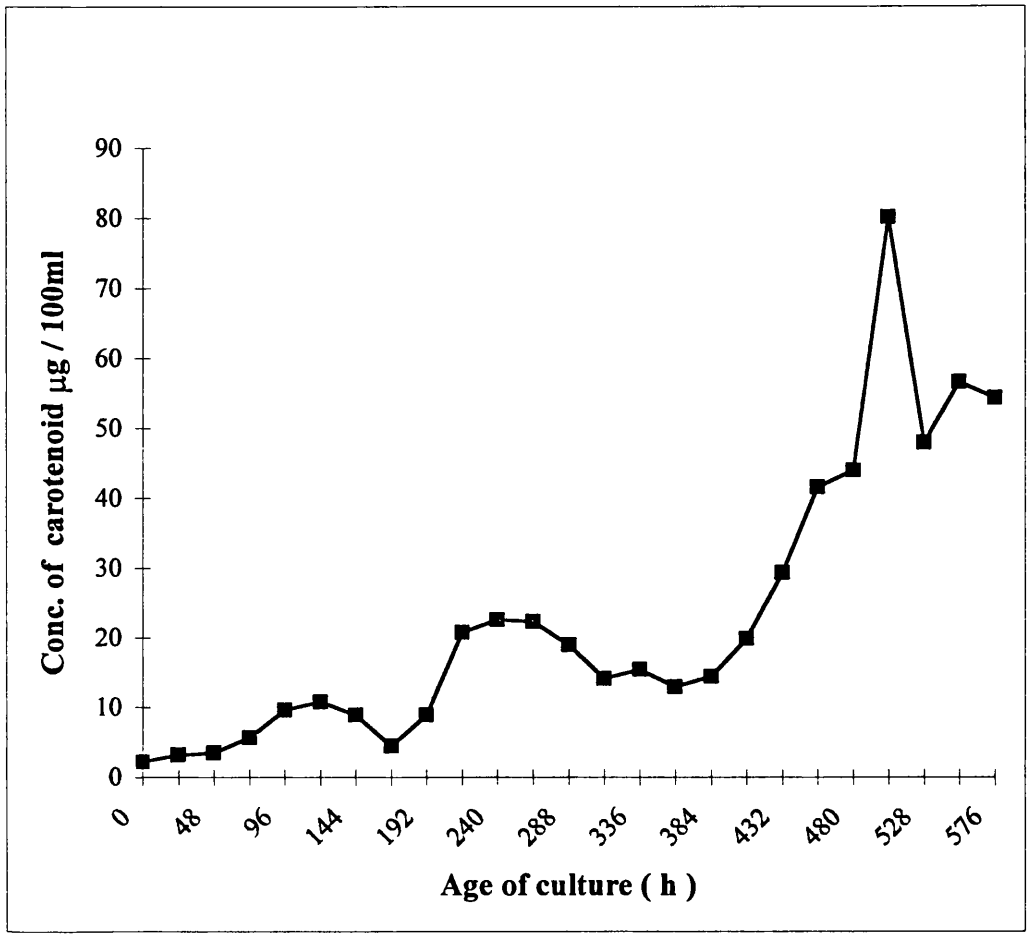


Fig 3.3 Concentration of carotenoid from *Aphanizomenon* sp. culture grown at 28° for 25 days.

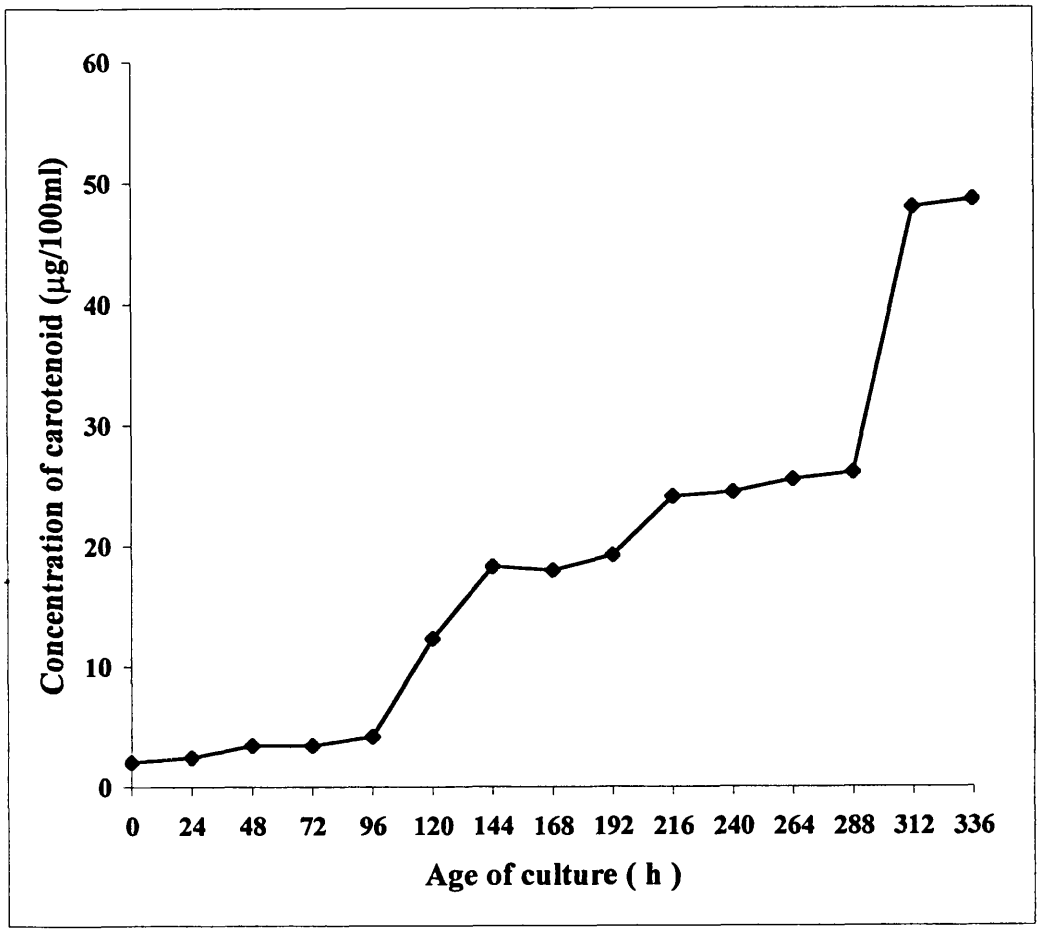


Figure 3.4 Concentration of carotenoid µg / 100ml from *Aphanizomenon* sp. culture grown at 28° for 14 days

subsequent increase in carotenoid concentration between 288h-312h, parallel to that seen in chlorophyll a concentration in the same culture (Fig 3.2).

3.2.c Growth curve Determination Based on Measurement of Dry Weight.

The growth of *Aphanizomenon* sp. was studied by determining the dry weight of cells recovered from duplicate 10ml aliquots of culture as described in Chapter 2.10. As shown in Fig 3.5, the dry weight did not increase during the first 120h after inoculation, after which period dry weight increased gradually between 144h and 192h, and then more rapidly between 216h – 336h. After this period however there was no further significant increase in dry weight. Thses observation suggest that the accumulation of chlorophyll a and carotenoid seen between 430h-600h (Figs. 3.1 and 3.2, respectively) may represent increased accumulation of these pigments within *Aphanizomenon* cells, rather than a significant increase in the number of cells present. When the experiment was repeated, a broadly parallel pattern was again seen in dry weight (Fig 3.6) and the chlorophyll a and carotenoid accumulation indicating the presence of an early lag phase (0h-ca 96h) a growth phase (96-0ca 92h), whilst there was no significant increase in dry weight associated with the sharp increase in pigment concentration seen between 264h and 312h.

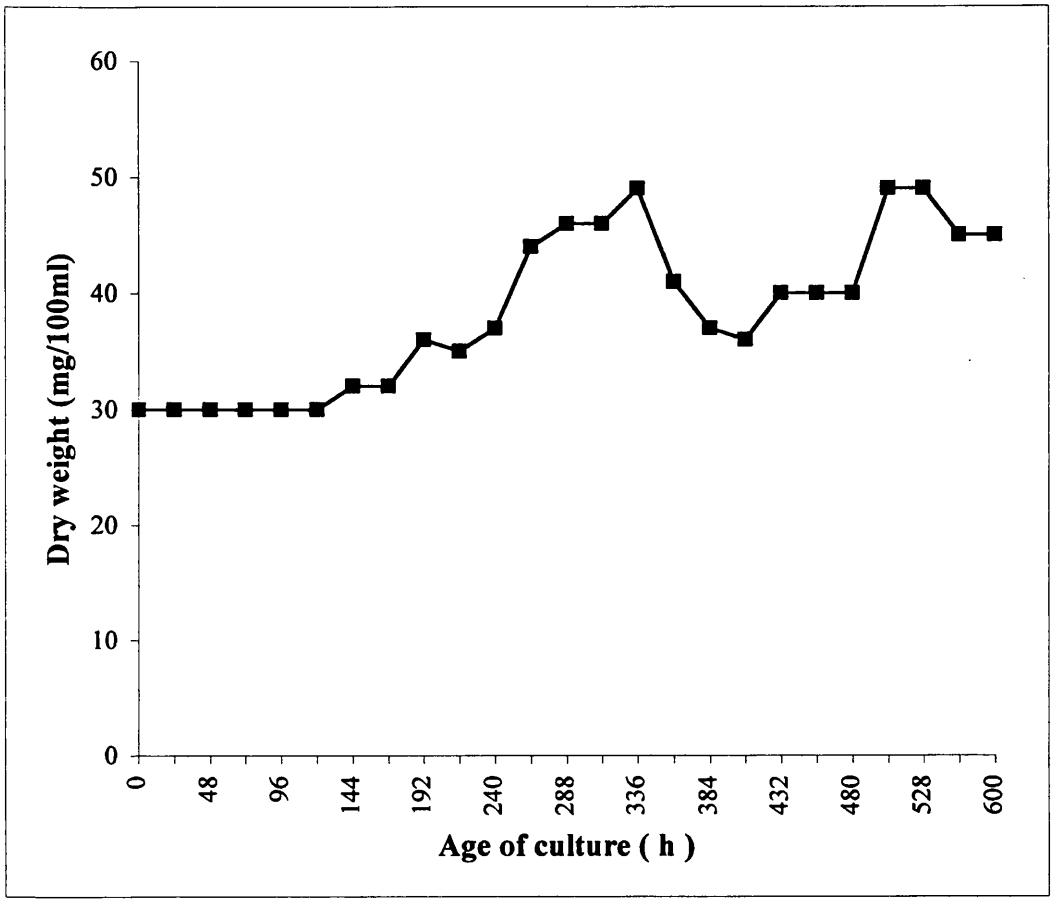


Figure 3.5 Dry weight of cells from *Aphanizomenon* sp. culture grown at 28° for 25 days

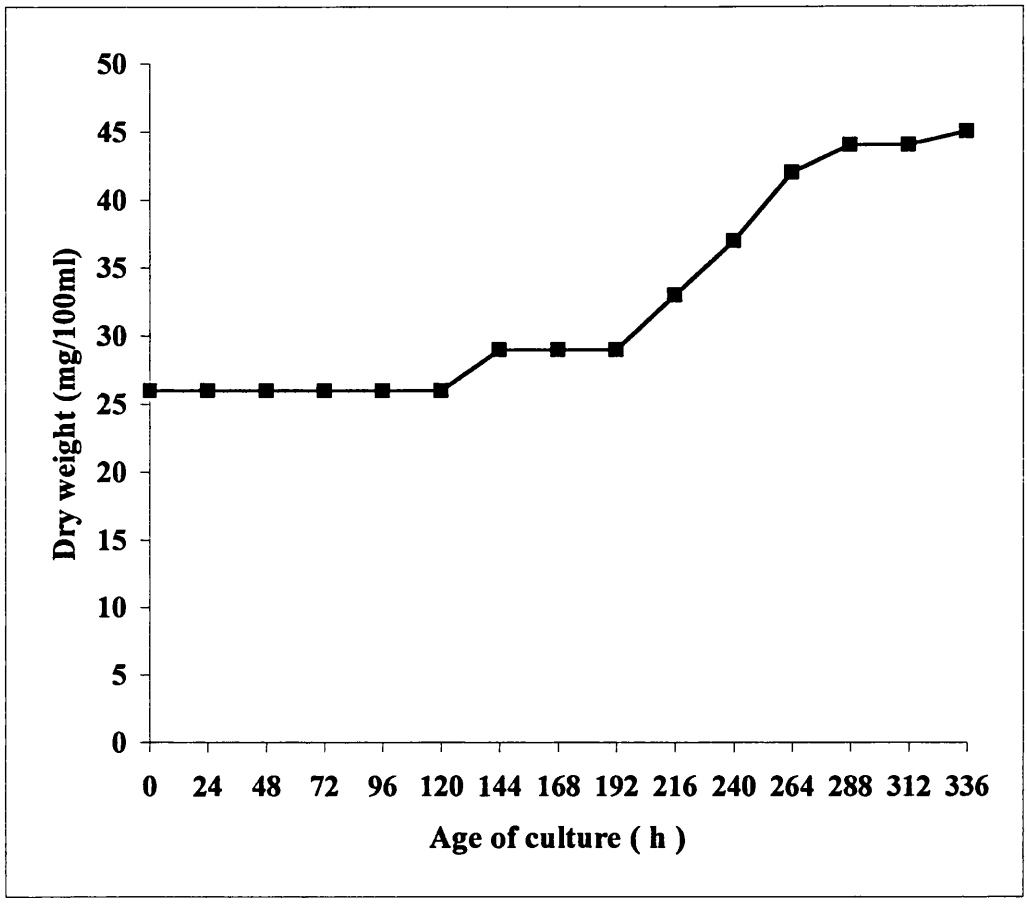


Figure 3.6 Dry weight of cells in *Aphanizomenon* sp. culture at 28° for 14 days.

3.3 Results of Studies on the Growth Characteristics of *Aphanizomenon* sp. Cultured at 15°

The growth characteristics of *Aphanizomenon sp.* cultured at 15° were determined in a parallel series of experments to these described above for culture grown at 28 °.

3.3.a Growth Curve Determination Based on Chlorophyll a Concentration of Cultures

The concentration of chlorophyll remained relatively constant for 168h after inoculation and then underwent a continuing substantial increase until the culture was 576h old (Fig 3.7). Growth under these conditions therefore showed a lag phase of identical duration (168h) to growth at 28°, followed at the lower temperature by an exponential growth phase, which was again followed by a second lag phase and then a further increase in chlorophyll a concentration.

3.3.b Growth Curve Determination Based on Carotenoid Content of Cultures

The growth curve based on carotenoid concentration (Fig 3.8) indicated a lag period between 0–148h, followed by an exponential phase of growth between 148–276h, and a second phase of sustained carotenoid accumulation was observed between 456h and 552h.

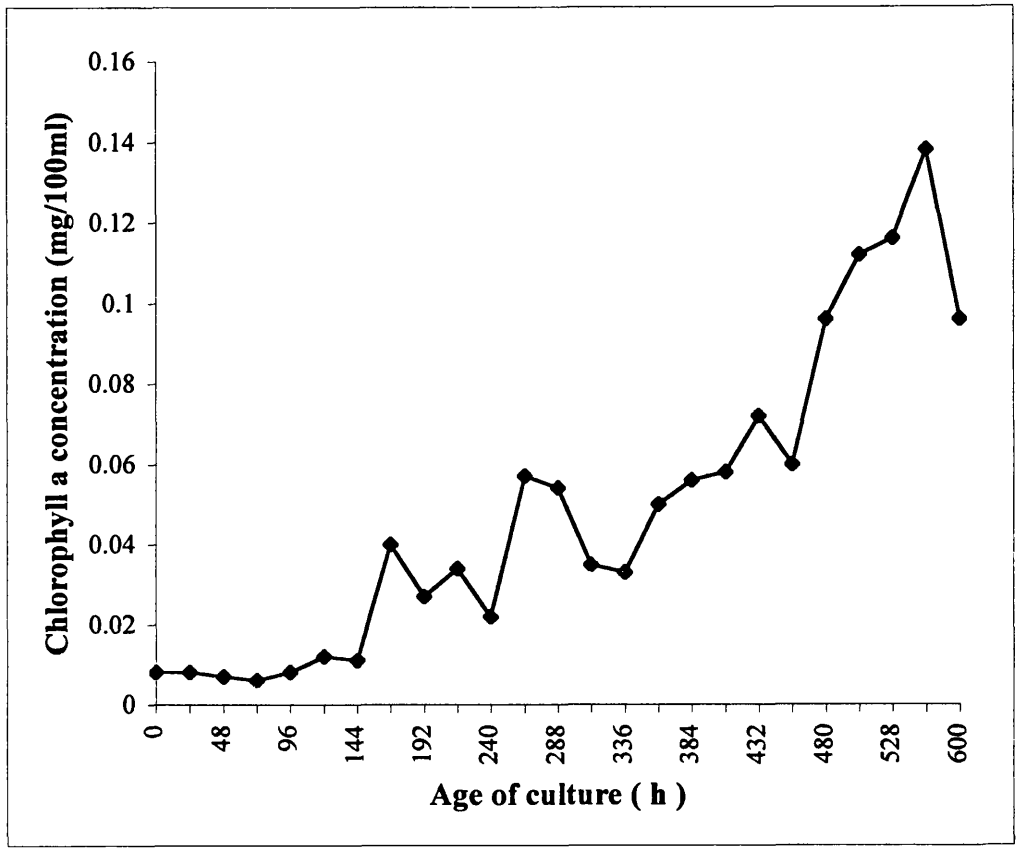
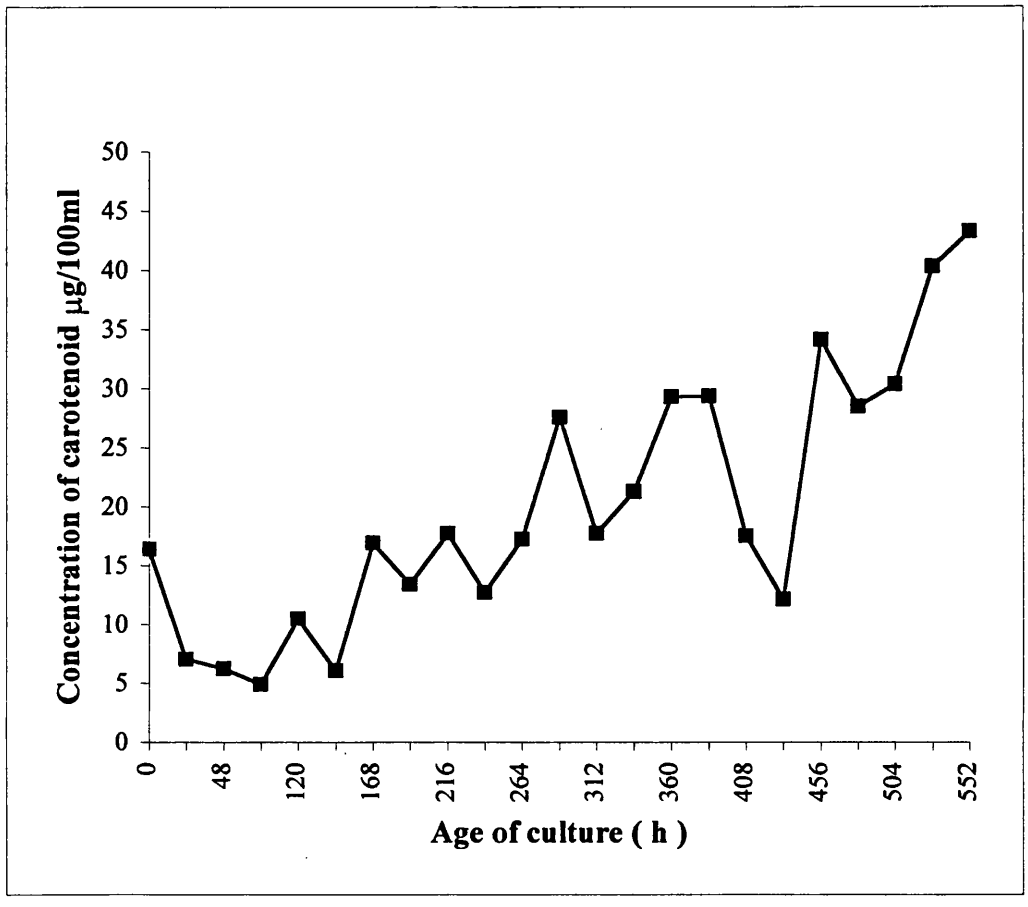


Figure 3.7 Chlorophyll a concentration from *Aphanizomenon* sp. cultures grown at 15° for 25 days.



Fiure 3.8 Concentration of carotenoid µg / 100ml from culture grown at 15° for 25 days

3.3.c Growth Curve Determination Based on Measurment of Dry Weight

The dry weight of the cells present in 100 ml (Fig 3.9) indicated an apparent lag period for 144h following inoculation, followed by an increase between 144 – 366h, after which the dry weight level remained relatively constant during an apparent stationary phase from 384 – 600h.

3.4 Discussion

The investigation of the growth characteristics of *Aphanizomenon sp* based on use of chlorophyll a levels, carotenoid concentration and dry weight as measures of biomass indicated that growth characteristics of the organism grown at either temperature were very similar. At both temperatures a lag phase of about 168h was observed after inoculation before the cultures entered their exponential phase. Under both conditions a doubling time of approximately 20h was observed, which although within the range reported for marine cyanobacteria (Mitsui *et al.*, 1986), was considerably shorter than the 66h previously reported (Jones, 1999) for *Aphanizomenon* sp. cultures grown in the same medium at 20° in aerated 20L batch cultures. These characteristics together with the observation that the maximum culture density observed at the two growth temperatures were very similar showing that the organism adapts its physiology to the different growth temperatures. The observation that the growth curves of *Aphanizomenon sp* grown at either temperatures indicate an accumulation of both chlorophyll a and carotenoid at the end of a stationary phase which is not associated with an increase in dry weight of the cells in the culture suggests this accumulation of pigments may be a response to

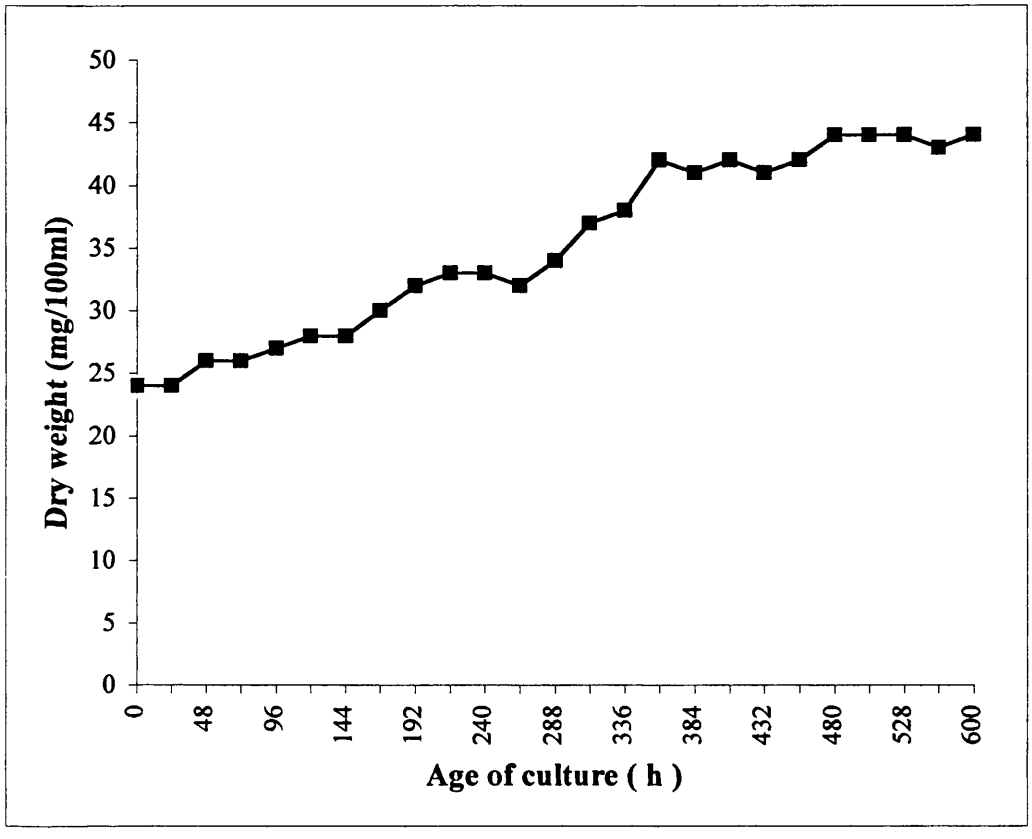


Figure 3.9 Dry weight of cells in *Aphanizomenon* sp culture grown at 15° for 25 days

stress, possibly associated with depletion of micronutrients. Changes in pigment composition in response to nitrogen deficiency have been observed in *Pseudoanabaena* sp and the *Oscillatoria splendida* (de Loura *et al.*, 1987). Another stress factor in the 28° culture, may be the prolonged exposure to the relatively high temperature (Vigh *et al.*, 1998). These studies also established that when grown at 28°, cultures between 10–12 days are typically in mid-late exponential phase of growth.

3.5 Fatty Acid Composition of *Aphanizomenon* sp. Grown in Laboratory Culture at 28° and at 15°

Cultures of *Aphanizomenon sp.* were grown for 25 days at either 28° or 15° and samples of cells were harvested at 24h intervals; the total lipid fraction was extracted according to the methods described in Chapter 2.5. Fatty acid methyl esters were prepared by transesterification and purified by preparative TLC (see section 2.6 and 2.7.1) respectively, and the fatty acid methyl esters were analysed by capillary GLC (see Section 2.8). Table 3.1 shows the relative percentage composition of fatty acids present over the time course when the organism was grown at 28° for 25 days. It was clear that in addition to the presence of saturated C-16 and C-18 fatty acids, both monoenoic and polyunsaturated fatty acids were present (Table 3.1). In the monoenoic fraction two C-18 isomers, 18:1(9) and 18:1 (11), together with 16:1(9), were identified by their relative retention times, (see Section 2.8) whilst the major polyunsaturated fatty acids were identified as 18:2 (9,12), 18:3 (9,12,15) and 16:3. Besides these major unsaturated fatty acids, a small amount of 16:2 was detected. No evidence of 18:3 (6,9,12), nor of polyunsaturated fatty acid containing 4 double bonds was found.

Table 3.1 Fatty acid relative percentage composition in *Aphanizomenon* sp. from cells grown at 28°.

Age of Culture (h)	Fatty Acids % Composition at 28°							
	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0	37.3	18.9	1.0	3.8	3.7	8.1	5.9	21.5
48	38.6	15.9	0.8	3.1	5.8	9.5	5.7	20.4
96	38.0	14.7	1.0	2.9	4.4	8.8	6.7	23.7
144	36.2	16.1	1.6	3.7	2.4	8.9	6.8	24.3
168	37.9	15.2	1.8	3.3	2.1	8.2	6.5	25.1
216	35.1	18.3	1.7	3.4	2.1	8.8	6.8	23.8
240	40.4	17.3	0.8	2. 5	4.2	12.4	5.2	17.3
264	41.5	18.4	1.1	1.9	2.3	10.4	6.1	18.3
288	36.9	17.3	1.7	3.6	2.4	8.1	6.7	23.4
312	36.5	20.3	1.7	3.0	2.1	8.2	6.2	22.0
336	37.8	19.1	1.5	3.3	2.4	8.5	6.1	21.3
384	33.5	24.6	1.8	2.5	2.2	10.8	5.2	19.4
432	32.2	25.0	1.3	2.5	2.8	12.4	5.7	18.2
480	35.5	28.2	1.1	2.2	2.6	10.8	4.1	15.7
552	35.5	27.6	1.0	1.2	3.6	15.7	4.7	10.7
600	33.0	29.2	0.9	2.0	3.5	14.5	4.1	12.8

The experimental data established that 16:0 was the most abundant fatty acid present throughout the 25 days of the study (Fig. 3.10). There was no significant increase in the proportion of 16:0 during the first 216h, but there appeared to be a small increase during the period from 216h-264h, after which the level of 16:0 started to slowly decline (336h-576h). There appeared to be a small gradual increase in the monoenoic fatty acids (16:1 and 18:1) between 216h and 336h, followed by a more pronounced increase after 384h. Having maintained an approximately constant level up to 312h, the proportion of 18:3 then showed a significant decrease. This loss of polyunsaturated fatty acid was accompanied by the conomitant increase in the proportion of monoenoic fatty acid (Fig 3.10). Although during the isothermal growth there were distinct changes in the fatty acid composition after about 312h, the fatty acid composition was relatively constant in the first 13 days of growth at 28°.

Table 3.2 shows the total percentage of the fatty acid compositions of *Aphanizomenon sp.* grown at 15 ° for 25 days. The cultures were found to contain the same complement of fatty acids as was present following growth at 28°. During the first 144h after inoculation there was a decrease in 16:0 and a concomitant increase in both 18:3 and 16:3 but there is no significant change in 16:1 in the same period (Fig 3.11). After this period of growth the fatty acid profile characteristic of culture at 15° appears to be established in which 18:3 predominates (35 to 40%) with substantial amounts of 16:3 (about 14%), whilst the relative level of 16:0 is about 23.5%. The observation described above clearly show that *Aphanizomenon sp.* modulates its membrane lipid fatty acid composition in response to growth temperature. At 28°, the relative level of saturated fatty acids is high during the entire growth period whilst, in contrast in the cells grown at 15°, the content of the

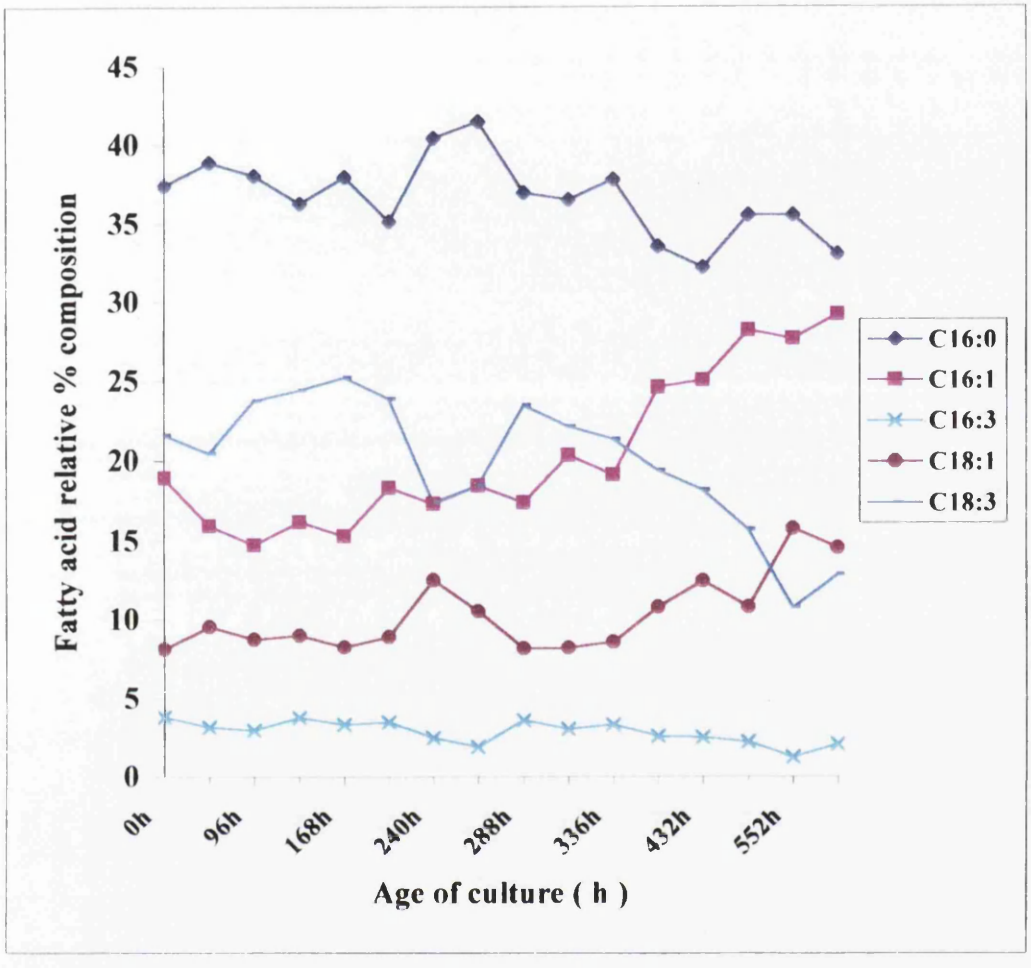


Figure 3.10 The level of the most abundant fatty acids present in *Aphanizomenon sp.* from culture grown at 28° for 25 days.

polyunsaturated fatty acid was high relative to the saturated fatty acids.

Comparison of the ratio of saturated: monoenoic: polyunsaturated fatty acid present after 240h growth at 28° (1 : 0.66 : 0.58) with that after 240h growth at 15° (1 : 0.66 : 2.13) indicated that the proportion of saturated to monoenoic fatty acid was unaffected by growth temperature, but that there was a 3.7 fold increase in the proportion of polyunsaturated fatty acid at the lower growth temperature. Within the polyunsaturated fatty acid fraction the increase was most marked in the trienoic compoments. An approximate doubling in the proportion of 18:3 was observed (from 18% to 39%), whilst the relative proportion of 16:3 increased some 6. fold (from 2% to 13%). These differences in fatty acid composition with growth temperature can be regarded as an adaptive response to changes in the ambient temperature associated with membrane remodeling and to alterations in the organization and dynamic properties of membrane lipids.

This first systematic study of the fatty acid composition of *Aphanizomenon sp* indicated that the organism should be classified as a Group 2 cyanobacterium as defined by Kenyon (1972) and Kenyon *et al.* (1972), as it contains 16:1,16:2,18:2 and α18:3 but not γ18:3 or 18:4, The fatty acid composition of *Aphanizomenon sp.* was similar to that of *Anabaena variabilis* (Sato *et al.*, 1979). It is interesting to consider how cyanobacteria perceive temperature and the nature of the signalling cascade which transduces lowered temperature to increased membrane fluidity. Experimental evidence has been presented which suggests that the change in fluidity of lipids in the plasma membrane caused by cold stress triggers increased expression of fatty acid desaturase systems, which leads to the desaturation of membrane lipids. (Vigh *et al.*, 1993). More recently it has been proposed that phase transition in microdomains of the plasma membrane leading to conformational changes affecting

Table 3.2 Fatty acid relative percentage composition in *Aphanizomenon* sp. from cells grown at 15°.

Age of Culture (h)	Fatty Acids % Composition at 15°							
	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0	32.7	12.9	0.9	2.2	9.2	15.6	8.5	18.0
24	25.4	17.3	2.2	7.5	3.1	7.5	6.3	30.8
72	29.5	16.5	2.6	8.3	1.9	7.1	5.6	28.5
120	25.9	15.1	1.9	11.8	1.4	5.1	3.3	35.4
168	23.7	15.8	1.8	12.2	2.6	4.6	3.3	36.0
192	26.4	12.9	1.7	13.3	1.3	4.7	2.9	36.8
240	25.1	12.7	1.8	13.3	1.3	4.7	2.9	38.5
264	25.1	13.1	1.6	12.6	1.3	5.0	2.7	38.6
288	25.9	12.5	1.5	12.3	1.4	5.3	2.2	38.9
336	23.5	11.2	1.7	15.1	0.8	3.9	2.4	41.2
364	25.5	11.4	1.6	14.0	1.0	3.8	2.8	39.9
432	23.3	13.0	1.7	13.9	1.0	5.0	3.0	39.1
504	23.4	12.4	1.9	14.4	0.7	4.5	3.1	39.7
552	23.5	12.9	1.9	13.6	0.9	4.5	3.1	39.4
600	24.1	10.3	1.8	13.4	0.9	4.5	3.9	41.0

a sensor protein may be the primary event in transduction (Murata and Los, 1997). In other organisms such as *Escherichia coli* (Marr and Ingraham, 1962), ciliates (Erwin and Bolch 1963), a yeast (Kates and Paradis,1973) and fungi (Summer *et al.*, 1969), only decreases in unsaturation are seen during isothermal growth when compared with cyanobacterial species.

The results of the present study of the effect of growth temperature on fatty acid composition in *Aphanizomenon sp* are in general agreement with those of Sato et al., (1979), who studied the fatty acid composition of total lipid from *Anabaena variabilis* grown isothermally at two different temperatures 38° and 22°. They reported that the average number of double bonds in a lipid molecule increased from 1.82 to 2.71 at the lower growth temperature, and that the most prominent effect was seen in the increased proportion of 18:3. In this organism 16:3 was not detected.

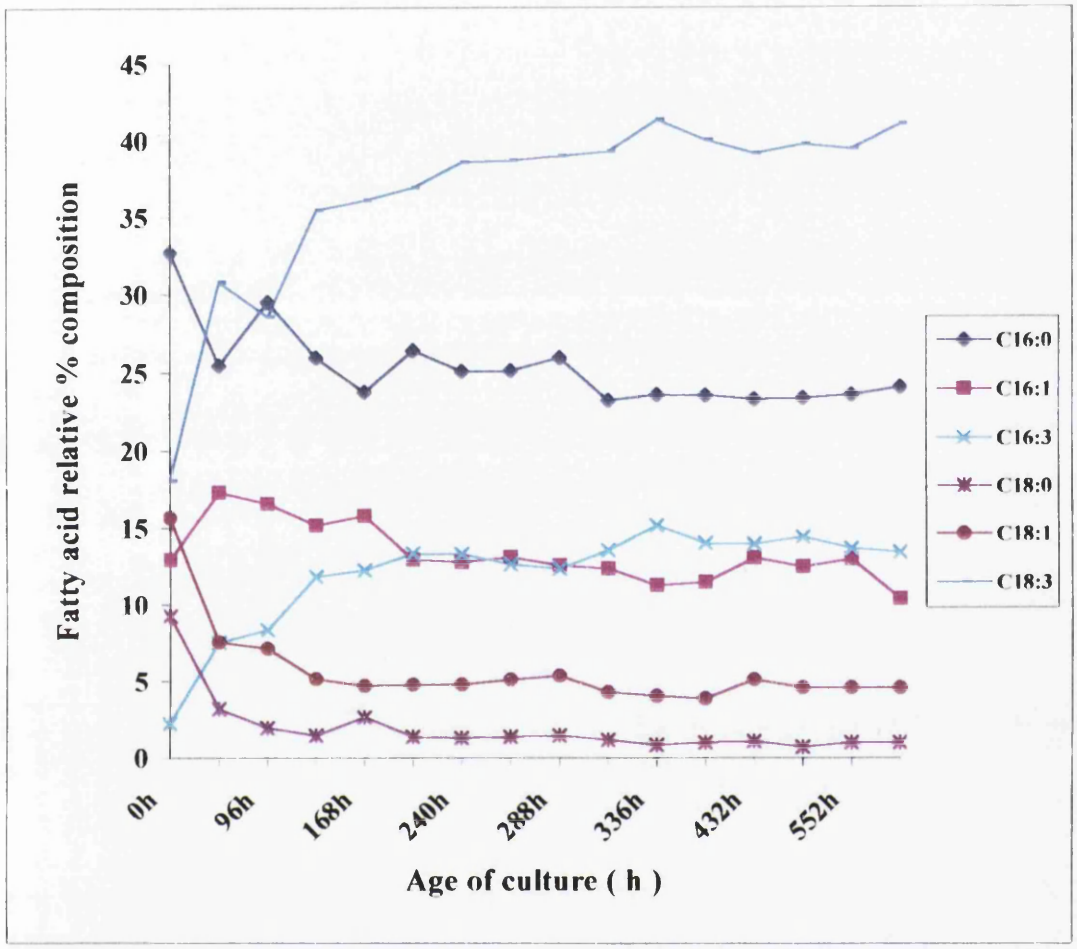


Figure 3.11 The level of the most abundant fatty acids present in *Aphanizomenon sp.* from culture grown at 15° for 25 days.

CHAPTER FOUR

Effect of Growth Temperature Transition on Fatty Acid Composition of Membrane Glycerolipids

4.1 Introduction

An organism may maintain the level of molecular motion or "fluidity" of its membrane lipids by regulating the number of double bonds in the fatty acids of these lipids (Russel, 1984). When the fluidity of membrane lipids is reduced by a decrease in temperature, cyanobacteria and plants respond by introducing double bonds into the fatty acids of lipids, so that the membrane returns to a more fluid state (Vigh *et al.*, 1993).

Fatty acid composition of many organisms is affected when their normal environmental growth temperature either decreases or increases (Kate and Hagen, 1964; Wada; 1990). These modifications in glycerolipid fatty acid composition represent adaptations of membrane fluidity that enable the organism to carry out normal physiological function at either decreased or increased enviromental temperature, as described in Chapter 2.11. Compositional analyses of cyanobacterial lipid have demonstrated that shifts in growth temperature lead to several types of change, the nature of which may depend on the species and the lipid class. (Sato *et al.*, 1979). For example cyanobacterial species in Group 1, characterized by the presence of only saturated and monounsaturated fatty acids, can introduce one double bond at the Δ9 position whilst cyanobacterial species in Group 2, which contain polyunsaturated fatty acids, introduce double bonds at Δ9, Δ12, Δ15 respectively (Murata *et al.*, 1992).

4.2 Changes in Fatty Acid Composition in *Aphanizomenon* sp Lipids Induced by a Reduction in Growth Temperature from 28° to 15°.

Analysis of total lipid extracts from *Aphanizomenon* sp by thin layer chromatography (see Chapter 2.7.2) showed the presence of four major glycerolipid classes, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SL) and phosphatidylglycerol (PG). This glycerolipid composition was similar to that previously described for cyanobacteria including *A. nidulans* (Holton *et al.* 1964; Nichols *et al.* 1965), *A. cylindrica* (Hirayama,1967) and *A. variabilis* (Murata and Sato,1978,1979). Cultures of *Aphanizomenon sp.* were grown at 28° for 10 days then the temperature was reduced to 15°, as described in Chapter 2.8. Cultures were maintained at 15° for 48h, during which time samples of culture were collected at 0h, (immediately before lowering the temperature), then at 12h, 24h, 36h, and 48h. The results shown in the figures in the following sections are representative of 2 individual studies, data sets for which are shown in the tables contained in Appendix 3.

4.2.1 Total Lipid Fraction

Figure 4.1 shows the relative % composition of the fatty acids from the total lipid extract of *Aphanizomenon sp.* over this time course. The temperature shift from 28° to 15° caused significant changes in the fatty acid composition of the total lipid. A prominent effect on fatty acid composition in response to lowering the growth

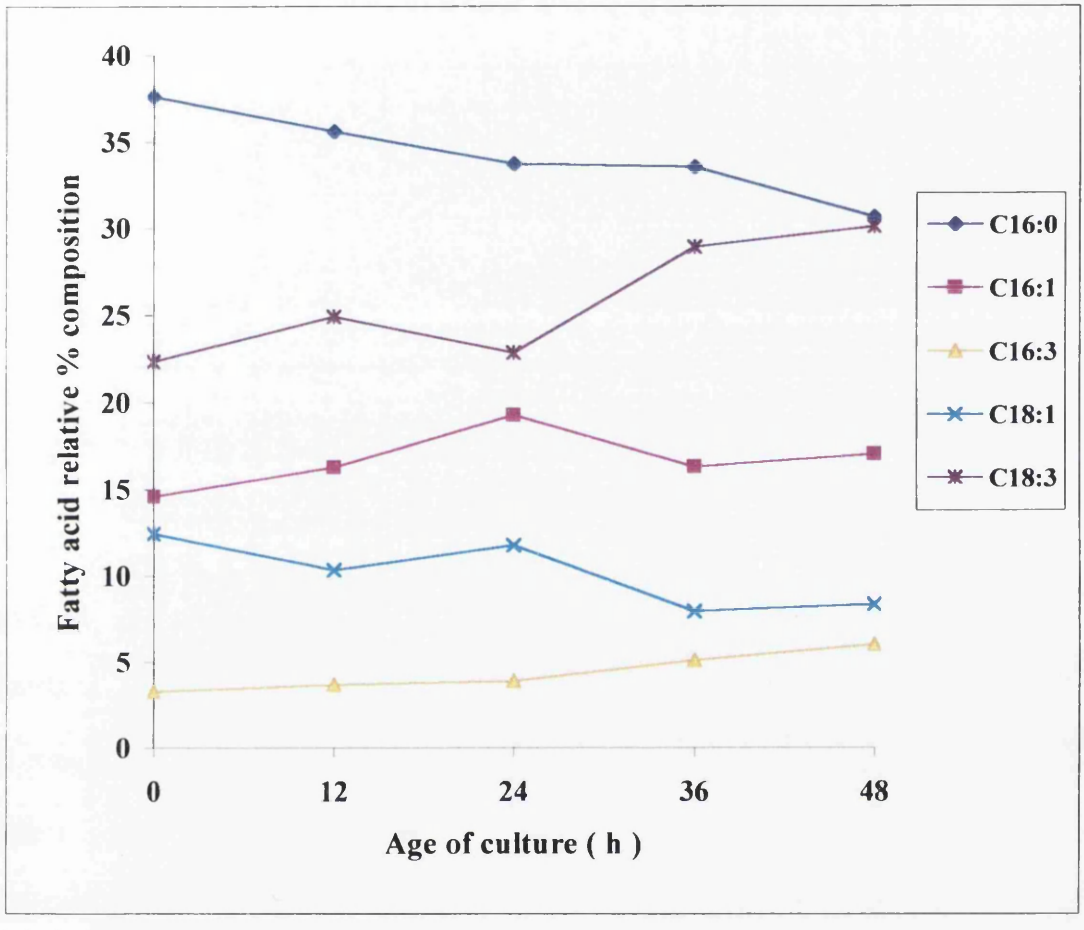


Fig 4.1 Relative % composition of the major fatty acids in the total lipid fraction from *Aphanizomenon* sp. culture following a temperature shift from 28° to 15°.

temperature was a decrease in the relative proportion of 16:0, which was 37.6% at the end of the growth period at 28°, and which fell to a level of 30.6% after 48h from lowering the temperature to 15°. This decrease in the level of saturated fatty acid was accompanied by an increase in the relative proportion of unsaturated fatty acids during temperature shift. The most prominent effect was an increase in the proportion of the polyunsaturated fatty acid 18:3, which had increased from 22% to 31% of total fatty acid after 48h at 15°. Over the same time period, the proportion of 18:1 was markedly decreased from 12.4% to 8.3%, and there was a concomitant decrease in the relative content of 18:2, which fell from 7.3% (0h) to 4.5% (48h); the level of 18:0 also fell from 2.3% at 28° to 1.6% after 48h at 15°. The level of 16:1 increased in the first 24h from 15% to 19%, and after that period the level of 16:1 started to decrease and remained almost constant. At 36h an increase in the relative proportion of 16:3 from 3% (0h) to 5% could be detected, and the increased level of 16:3 was sustained to the end of the sampling period. A possible explanation for the transient increase in the relative proportion of 16:1 between 12-24h at 15° may be that 16:1 formed during this period is successively desaturated to 16:3, which increased between 24-36h, perhaps reflecting induction of desaturases required for PUFA formation. However no significant pattern in the proportion of 16:2 was seen during the period of the temperature transition. The total proportion of fatty acids C-16 remained almost unchanged relative to total C-18 fatty acids during the temperature transition. In conjunction with the observed decreases in the more saturated fatty acids and the increases in the more unsaturated fatty acids, within both the C-16 and C-18 pools, these results are consistent with the view that pre-existing 16:0 fatty acid may be directly desaturated to yield the unsaturated C-16 fatty acids and that pre-existing 18:1 may be directly desaturated to 18:3.

4.2.2 Monogalactosyldiacylglycerol

To characterize the contribution of each glycerolipid class to the changes in fatty acid composition observed in the total lipid fraction following temperature shift from 28° to 15°, each of the major glycerolipid fractions was isolated from total lipid extracts from the culture during the time course of the temperature transition, and the fatty acid composition of each individual lipid class determined.

In the MGDG fraction (Fig. 4.2) the proportion of 16:0 decreased progressively during the first 36h at 15°. The substantial (38.3%) decrease observed at 36h appeared to be largely reversed between 36h and 48h. The level of 16:1 in MGDG did not show any clear trend on either of the 2 occasions this experiment was carried out. Whilst there was no significant change in the level of 16:2 over the time period of the study, the relative proportion of 16:3 had almost doubled to reach 8.7% after 24h at the lower temperature, and this level was sustained over the remainder of the time course. Amongst the C-18 fatty acids the most prominent effect was seen in a dramatic increase in the proportion of 18:3 which represented only 27.6% of the total fatty acid at 28°, but after 48h from the temperature shift to 15°, had increased to 37%. In this time period concomitant decreases in 18:0, 18:1, and 18:2 levels were observed (Fig 4.2 and Table 4.1 Appendix 3). These results contrast with the effects of a temperature down shift from 38° to 22° in *A. variabilis* (Sato and Murata, 1980a) in which the most marked change occurred in the C-16 fatty acids of MGDG, where after 10h 16:0 decreased and 16:1 increased, but the content of these fatty acids returned to almost the original level in the following 40h. An increase in the level of 18:3 was also evident in *A. variabilis* during 'high' to 'low' temperature transition within 10h, and the increase continued for at least 40h. It therefore appears that the temperature transition response observed in the MGDG fraction of

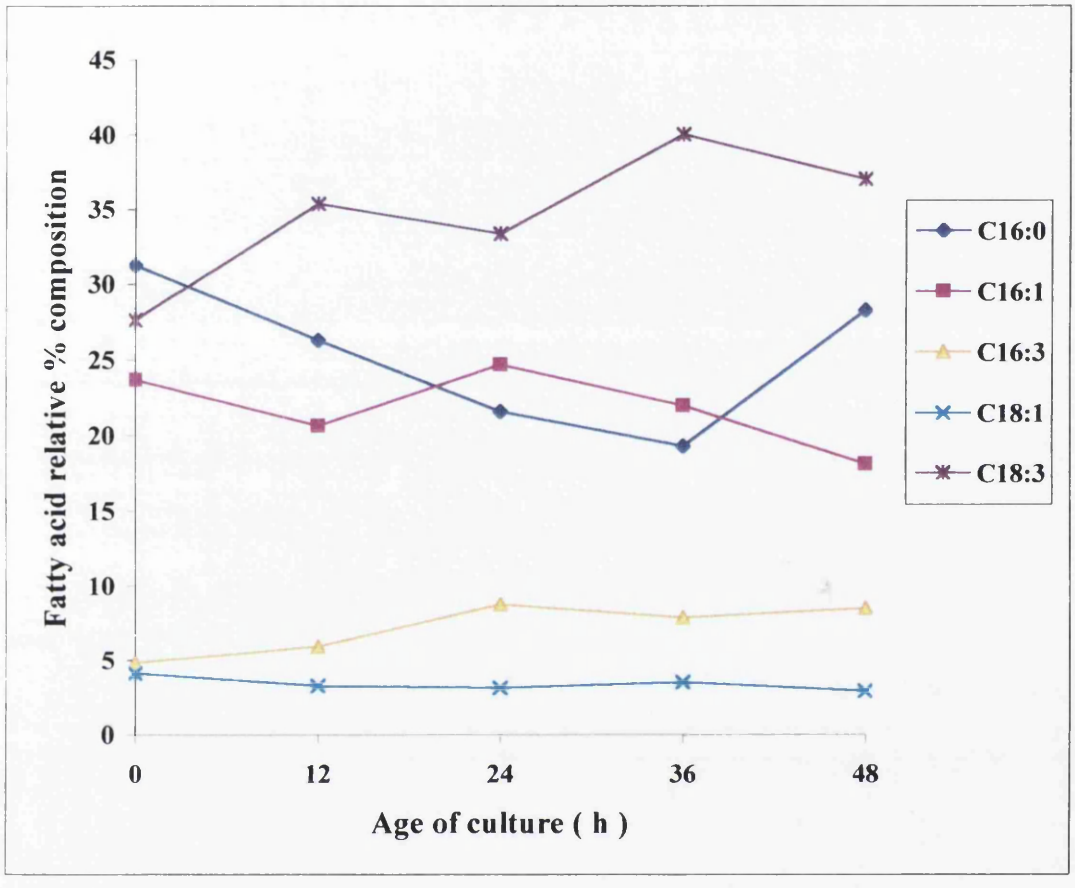


Fig 4.2 Relative % composition of the major fatty acids in the MGDG fraction from *Aphanizomenon* sp. culture following a temperature shift from 28° to 15°.

Aphanizomenon sp is distinct from that in *A. variabilis*, as no significant increase in unsaturated C-16 fatty acids was seen during the first 10h of temperature adaptation in the present study. Thus *Aphanizomenon* sp appears to lack this relatively rapid response which was proposed as central in temperature adaptation in *A. variabilis* (Sato and Murata 1980), and suggests the adaptive response within the MGDG fraction of *Aphanizomenon* sp is restricted to a relatively long term increase in 18:3 and 16:3 content; changes in C-16 fatty acids did not occur in the other lipid classes whilst the desaturation of the C-18 fatty acids was observed in the other lipid classes.

4.2.3 Digalactosyldiacylglycerol

In the DGDG fraction the level of 16:0 was relatively constant in the first 36h, after which there appeared to be a significant increase between 36 and 48h (Fig 4.3) Over the same time period, the concentration of 16:1 decreased from 16.4% at 28° to reach after 48h 11.5% at 15° . As was the case in MGDG, there was no consistent change in the proportion of 16:2 (Table 4.1 Appendix 3), but in contrast to the MGDG fraction, the 16:3 level in DGDG remained almost constant during temperature transition. Changes in the relative proportion of C-18 fatty acids were however marked. When the growth temperature was lowered from 28° to 15°, the relative proportion of 18:3 increased from 29.4% to 37.1% within 36h from the onset of the temperature shift after which it returned to its original level. The relative content of 18:1 showed little change over the same time period, and the proportions of 18:0 and 18:2 were also constant (Table 4.1 Appendix 3). The adaptive response in *Aphanizomenon* sp therefore appears to be broadly similar to that reported in *A.variabilis* (Sato and Murata, 1980) in which the most prominent response was a

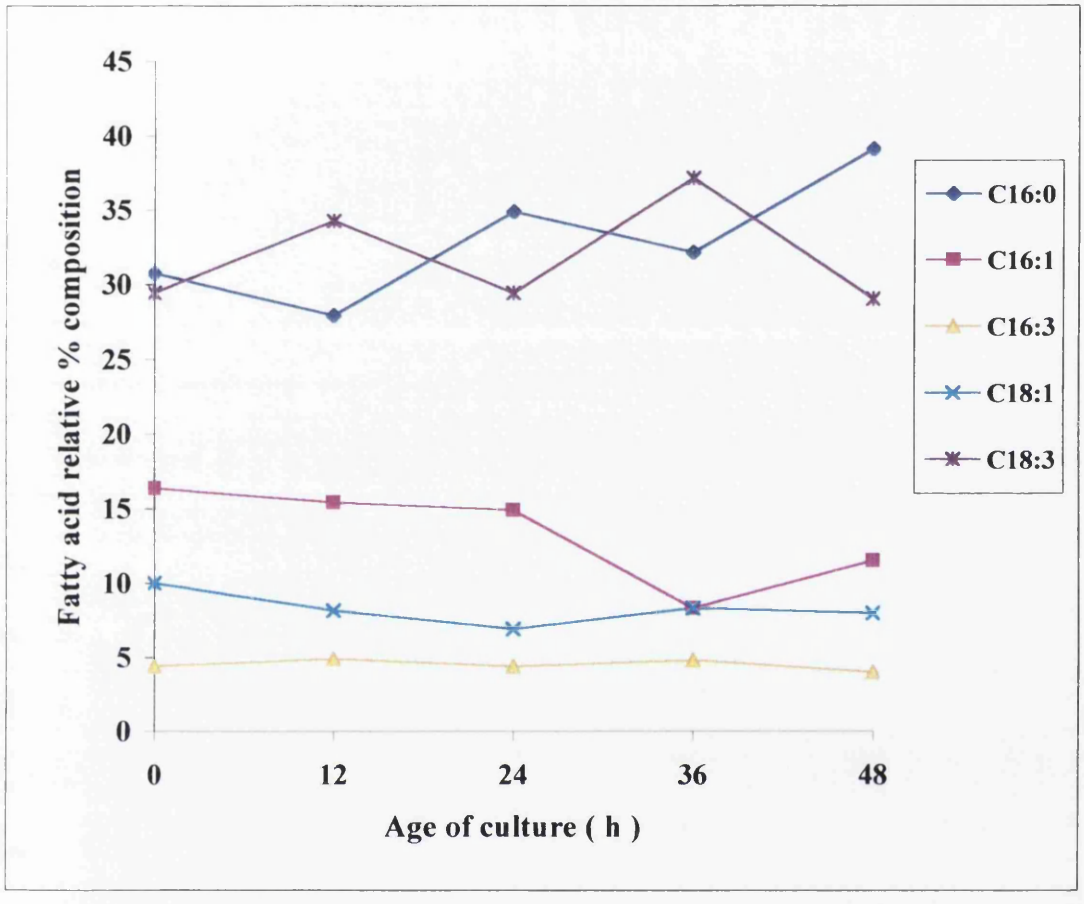


Fig 4.3 Relative % composition of the major fatty acids in the DGDG fraction from *Aphanizomenon* sp. culture following a temperature shift from 28° to 15°.

sustained increased in the proportion of 18:3 between 10 and 40h after the temperature transition, whilst there was a little change in the C-16 fatty acids.

4.2.4 Phosphatidylglycerol

The fatty acid composition of the PG fraction was characterized by the absence of 16:2 and 16:3 (Table 4.1 Appendix 3); the most abundant fatty acids present in cultures grown at 28° were 16:0 and 18:3, as was the case for the galactoglycerolipids. A marked change was observed in this phospholipid fraction in the C-16 fatty acids, where the proportion of 16:0 decreased by from 35% to 26% during the first 12h at low temperature, during which this period there appeared to be a small increase in the proportion of 16:1 and between 12h and 24h the level of 16:1 increased to 53% of its starting value. In this lipid fraction, the relative proportion of 18:1 had increased 2.5 fold within 12h of the temperature being reduced from 28° to 15°, whilst during the same time period the proportion of 18:3 was approximately halved, from 29% to 13% (Fig. 4.4). No significant trend was apparent in the proportion of either 18:0 or 18:2 during temperature transition (Tables 4.1 and 4.2, Appendix 3).

The effect of the reduction in growth temperature on the fatty acid composition of PG was quite different from that observed in the other three lipid classes. Since 16:2 and 16:3 appear to be absent from this lipid class in *Aphanizomenon sp*, desaturation of C-16 fatty acids in PG appears to be restricted to the formation of 16:1. The observation that 18:3 levels were decreased and that 18:1 levels increased substantially when the temperature was reduced from 28° to 15°, was in contrast to the effect observed in the galactolipids MGDG and DGDG, and appears to indicate

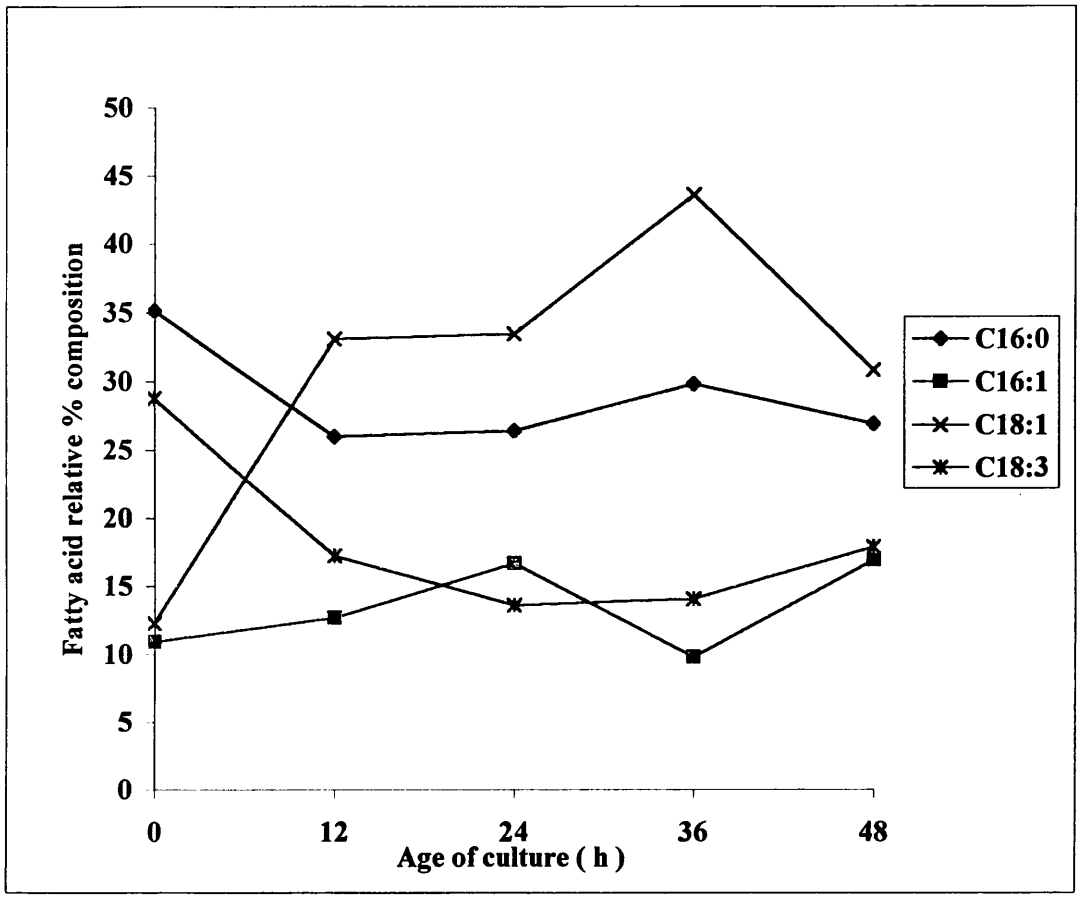


Fig 4.4 Relative % composition of the major fatty acids in the PG fraction from *Aphanizomenon* sp. culture following a temperature shift from 28° to 15°.

that *de novo* synthesis of 18:1 may be an important adaptive mechanism in retailoring this lipid class. The response in the PG fraction of *Aphanizomenon* is quite distinct from that reported in *A.variabilis* (Sato and Murata, 1980), where no significant change was seen in the C-16 fatty acid components during temperature transition, and 18:1 levels decreased whilst 18:3 levels increased.

4.2.5 Sulfoquinovosyldiacylglycerol

The fatty acids of the SL fraction were more saturated than those of the other glycerolipid fraction (Tables 4.1, 4.2 Appendix 3). It can be seen that the major fatty acid of this acidic glycerolipid was 16:0, and that there was a relatively small proportion of 18:3 in cultures grown at 28°. Whilst reduction of growth temperature had little effect on the proportion of 16:0 present (Fig 4.5), the relative level of 16:1 had increased from 8.3% at 28° to 16.5% after 36h at 15°, and whilst neither 16:2 nor 16:3 could not be detected in SL formed during culture at 28°, after 36h at 15° a small amount of 16:3 could be found (Table 4.1 Appendix 3). The major C-18 fatty acids in this fraction were 18:1 and 18:3. Reduction of the growth temperature produced a decrease in 18:1 level from 17.9% at 28° to 11.2% after 48h at 15°, whilst the proportion of 18:3 increased over the same time period from 11.9% (28°) reaching 17.2% within 12h at 15°, after which the relative concentration of 18:3 remained constant over the remaining time course of experiment. This result was in contrast to the adaptive changes occuring in the SL fraction of *A. variabilis* in which, whilst there was a substantial increase in the level of 18:3, there was no significant change in the C-16 fatty acids which contain only 16:0 and 16:1 (Sato and Murata 1980a).

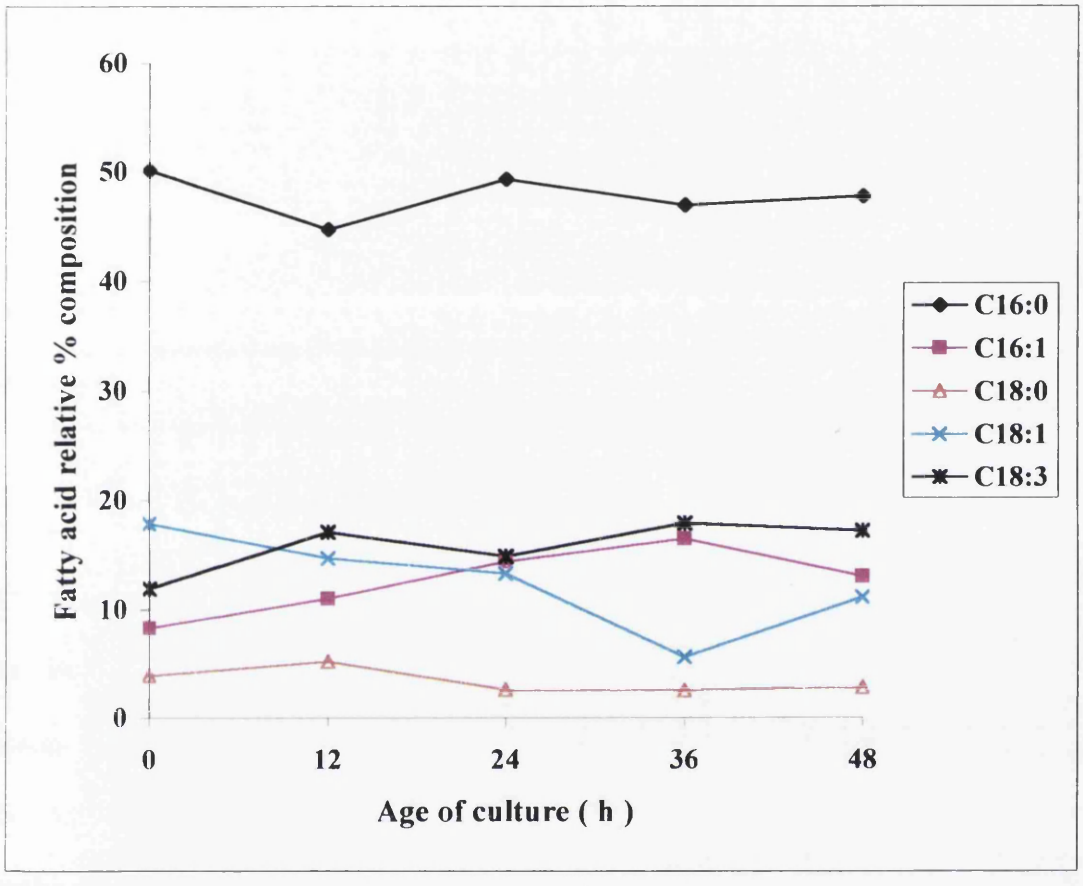


Fig 4.5 Relative % composition of the major fatty acids in the SL fraction from *Aphanizomenon* sp. culture following a temperature shift from 28° to 15°.

4.3 Discussion

The present study indicated that the fatty acid composition of the three lipid classes MGDG, DGDG and SL respond to lowering the growth temperature in relatively similar ways, while PG responds to low temperature in a quite different way. Moreover, when the temperature decreased, in *Aphanizomenon sp.* the amount of more unsaturated fatty acids increased at the same time that the amount of more saturated fatty acids decreased. However, during the present work it was found that in spite of the changes obtained in the fatty acid composition following the temperature shift, the total amounts of C-16 and C-18 acids were almost constant.

MGDG and DGDG had similar fatty acid composition and contained polyunsaturated C-16 and C-18 fatty acids, whilst in the acidic glycerolipids PG and SL there was no polyunsaturated C-16 fatty acid, and the level of 18:3 was generally lower than in the galactolipid fractions in cultures grown at 28°. The present study indicates that the fatty acid composition of total lipid in *Aphanizomenon sp.* changes following a transition from 'high' to 'low' growth temperature. Lowering of the growth temperature from 28° to 15° led to several changes in the relative proportion of the saturated and unsaturated fatty acids present in the four major glycerolipid classes MGDG, DGDG, PG and SL. The changes observed in the fatty acid composition of the total lipid after lowering the temperature were broadly similar to those observed in the MGDG and DGDG fractions consistent with the quantitative preponderance of these glycerolipid classes. Reduction of the growth temperature led to a decrease in 16:0 and 18:1, while the contents of the polyunsaturated fatty acids 16:3 and 18:3 increased as summarised in Table 4.3. In contrast, the trend of the changes in fatty acid composition of PG was quite distinct. In this lipid class the level of the monoenoic fatty acids 16:1 and 18:1 increased, whilst the level of 18:3

Table 4.3 Changes in fatty acid relative % composition of lipid fractions from *Aphanizomenon* sp following temperature transition from 28° to 15° for 36h.

Fatty acid increase (↑) or decrease (↓)

Lipid fraction	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3
Total lipid	↓	↑	↓	↑↑↑	↓↓↓	↓↓↓	↓↓↓	↑↑↑
MGDG	-	↑↑	-	↑↑↑	↓↓↓	↓↓	↓↓↓	↑↑↑
DGDG	↑↑	↓↓	-	-	↓↓	↓↓	↓	-
PG	↓↓	↑↑↑	nd	nd	↓	↑↑↑	↓↓↓	↓↓↓
SL	-	↑↑↑	nd	nd	↓↓	↓↓↓	↓↓	↑↑↑

↑↑↑	(≤ 30%)	↓	(≤ 10% decrease)
↑↑	(≤ 20%)	↓↓	(≤ 20% decrease)
↑	≤ 10%	↓↓↓	(≤ 30% decrease)
-	= 0 - 10		
nd	Not detected		

decreased (Table 4.3). In the SL fraction 16:0 was the dominant fatty acid and its level appeared to decrease slightly whilst 16:1 increased, and a fall in 18:1 levels was accompaned by an increase in the proportion of 18:3. A significant observation in the current study was the dramatic increase in the proportion of 18:3 in response to reduced temperature which appears to be specific to the MGDG fraction. The substantial replacement of 16:0 by 16:3 may significantly increase the fluidity of the photosynthetic membrane system generally, or perhaps be important in modulating the fluidity of specific micro-environments within the membrane system.

From these results, it was concluded that there is a positive relationship between a temperature down-shift and the fatty acid % composition of *Aphanizomenon sp.* Moreover, the experimental results clearly demonstrate the presence of relatively rapid and slow responses in the mechanisms by which the fatty acid content is modulated during temperature acclimation. Relatively rapid responses (occuring within 12h) were represented by a decrease in 16:0 and a concomitant increase in 18:3 in MGDG and DGDG. A substantial rapid response, limited to the first 12h following temperature transition was observed in the PG fraction in which 18:1 increased almost 2.5 fold, whilst 16:0 and 18:3 level decreased (Table 4.3). Neither of these phenomena can be readily explained by direct desaturation of fatty acyl chains in pre-existing galactolipid or PG molecules. A slower sustained response was observed in the accumulation of 18:3 in the total lipid, MGDG and SL fraction (Table 4.3) and the accumulation of 16:3 specifically in MGDG, which may be indicative of direct desaturation of glycerolipid bound fatty acyl chains concomitant with a decrease in 18:1 and 16:0 respectively.

The responses seen in the glycoglycerolipids C-16 fatty acids of *Aphanizomenon* sp are in contrast to those reported by Sato and Murata (1980a), who

found that in the first 10h after a temperature shift from 38° to 22° in *A. variabilis*, most of the 16:0 of the total lipid and MGDG was desaturated to 16:1; in the following hours, the relative contents of 16:0 and 16:1 were almost restored to original levels. Also in *A. variabilis*, 18:0 and 18:1 appeared to be desaturated to 18:3 in all lipid classes whereas in *Aphanizomenon* the 18:3 content of the PG fraction decreased substantially following the temperature downshift.

The results in the present work are also in contrast of those reported by Evans *et al.*, 1996. In a preliminary study on *Aphanizomenon flos-aquae* restricted to an analysis of total lipid fatty acid these workers reported that following a temperature transition from 20° to 15° a rapid increase in the proportion of 16:1 occurred in the total lipid extract, but thus did not detect an increase in 18:3. It may be that the relatively small temperature transition used in this study was insufficient to trigger the sustained accumulation of 18:3 induced by the larger temperature shift used in the present study. MGDG appeared to be the most significant lipid class in *Aphanizomenon* sp, showing responses in both its C-16 and C-18 fatty acid composition during temperature adaptation. Thus in the following chapters MGDG will receive particular attention with respect to composition and metabolism..

The increase in desaturation of fatty acid in response to lowered growth temperature is predicted to result in a downward shift of the temperature at which the transition between the liquid crystalline and phase separation state of the membrane lipid occurs (Sato and Murata 1980a). These changes in fatty acid composition are interpreted in terms of regulation of membrane fluidity for the proper function of cyanobacterial membranes for example in photosynthesis (Ono and Murata 1979). During adaptation to a downward shift it therefore appears that *Aphanizomenon* sp increases the fluidity of its membranes by increasing the degree of unsaturation in the

fatty acyl chains of its glycerolipid, as previously observed in other cyanobacteria, but that the processes by which the desaturation is brought about may be different from those in other cyanobacterial species (Holton *et al.*, 1964; Sato *et al.*, 1979).

4.4 Changes in Fatty Acid Composition of *Aphanizomenon sp* Lipids Induced by an Increase in Growth Temperature From 15° to 28°

Fatty acid compositions of *Aphanizomenon sp.* lipid fractions following an upward temperature shift from 15° to 28° were determined as described in Chapter 2.8. The organism was grown at 15° for 10 days, then the temperature of the incubator was raised to 28° and growth continued for 48h. Samples of culture were collected at the time the temperature was raised (0h) to 28° and after further periods of 12h, 24h, 36h, and 48h. The Figures shown in the following sections are representative of 2 individual studies, data sets for which are shown in the tables in Appendix 3.

4.4.1 Total Lipid Fraction

Increasing the temperature from 15° to 28° resulted in changes in the fatty acid % composition of the total lipid fraction (Fig. 4.6). The most prominent effects occurred in the 16:1 and 18:1 components, of which the relative concentration of each increased in parallel and had doubled by the end of the transition period. No significant effect on these fatty acids could be detected after 12h, the onset of accumulation occuring between 12-24h, indicating a relatively slow adaptive response (Fig. 4.6). Concomitant with this increase in the C-16 monoenoic fatty acid,

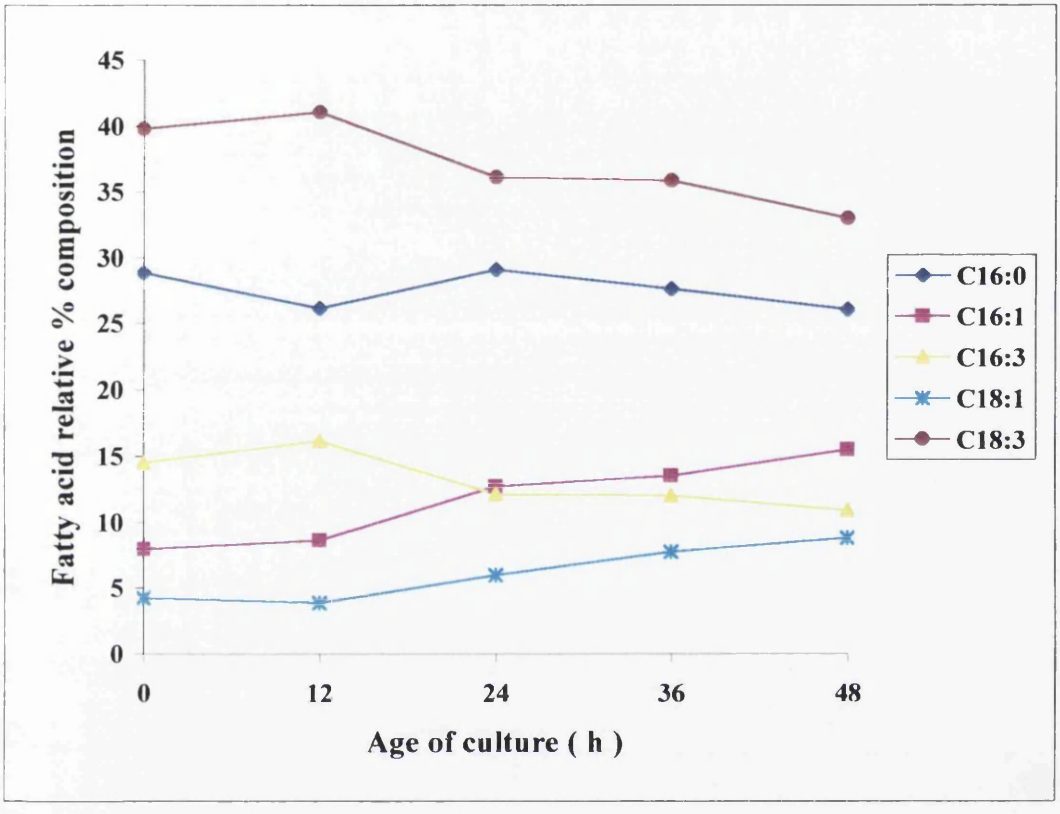


Fig 4.6 Relative % composition of the major fatty acids in the total lipid fraction from *Aphanizomenon* sp. culture following a temperature shift from 15° to 28°.

a significant decrease in proportion of 16:3 was observed and the level of this fatty acid had declined from 15% to 11% by the end of the transition period. As well as the substantial changes in this C-16 fatty acids, a parallel decrease in 18:3 occurred at the same time as the progressive increase in the relative proportion of 18:1. In contrast the 16:0, 18:0 and 18:2 content was relatively constant throughout the transition period. The observed pattern of changes in a fatty acid composition is consistent with the onset of *de novo* biosynthesis of monoenoic fatty acids occurring between 12-24h after the temperature transition and the replacement of the pre-existing polyunsaturated fatty acids.

4.4.2 Monogalactosyldiacylglycerol

Following the temperature shift from 15° to 28°, the proportion of 16:1 in MGDG approximately doubled from that present in cells grown at 15° (8%) to reach a level of 16.3% after 48h, whilst over the same time period, there was a reduction in the relative amount of 16:3, but no change in the proportion of 16:0 (Fig. 4.7); only trace amounts of 16:2 (Table 4.4 Appendix 3) could be detected. Over the same time period the levels of 18:0 and 18:2 were unchanged (Table 4.4, Appendix 3) whilst the level of 18:3 declined slightly. In parallel with this decrease in 18:3, the proportion of 18:1 appeared to increase over the time course of the transition. Generally the pattern of changes in fatty acid composition seen in the MGDG fraction was consistent with that observed in the total lipid fraction.

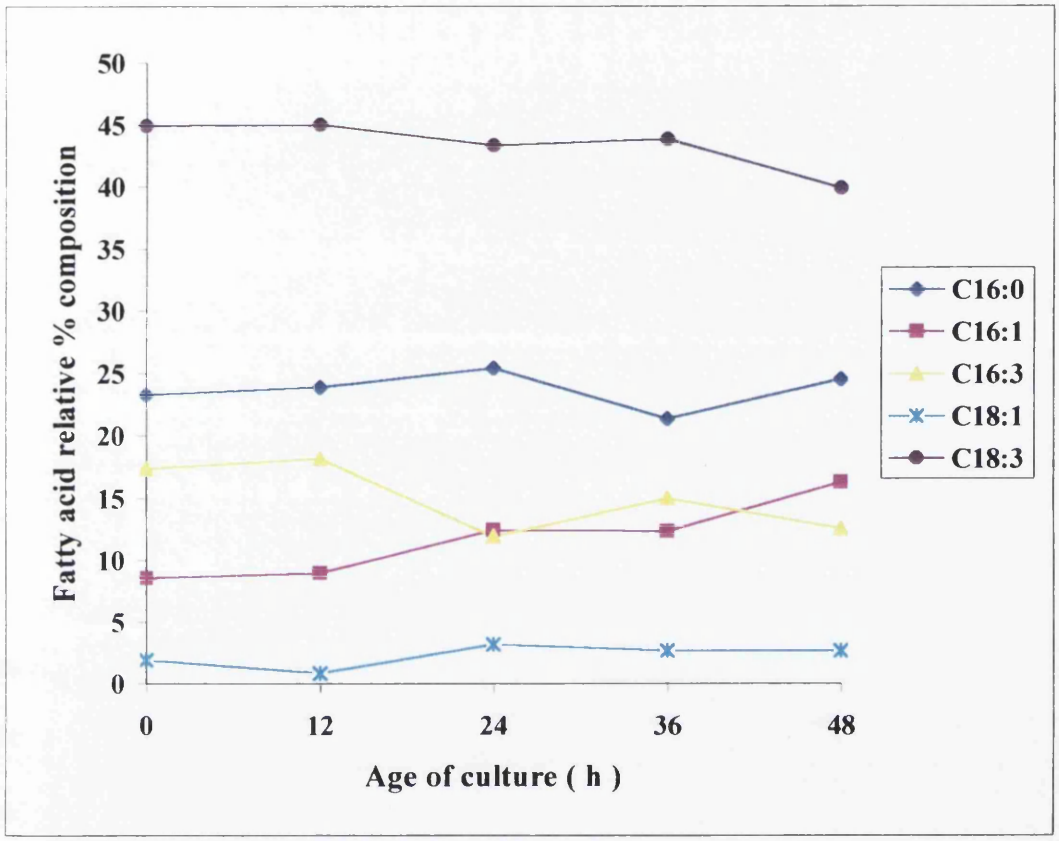


Fig 4.7 Relative % composition of the major fatty acids in the MGDG fraction from *Aphanizomenon* sp. culture following a temperature shift from 15° to 28°.

4.4.3 Digalactosyldiacylglycerol

In this lipid fraction upward temperature shift resulted in an 18.3%, increase in the proportion of 16:1 (Fig. 4.8), which was accompanied by a small apparent decrease in the proportion of 16:0 from 30.9% to 26.9%, but in contrast to the MGDG fraction, there was no significant decrease in the relative amount of 16:3 over the same time period. Also in contrast to the MGDG fraction, there was little significant change found in proportion of any C-18 acid following the upward temperature shift.

4.4.4 Phosphatidylglycerol

The most prominent effect in this lipid class on fatty acid composition in response to raising the growth temperature was an increase in the relative proportion of 16:1, which after 24h reached a level approximately 2 fold that present in cells grown at 15° (Fig. 4.9), whilst over the same time period, the content of 16:0 showed a parallel decrease. Whilst a small amount of 16:2 and 16:3 was found in this fraction from cultures grown at 15° before the temperature shift (0h), within 12h of the upward temperature shift 16:2 could not be detected, and after 24h 16:3 could not be detected. In contrast to the DGDG fraction marked changes were observed in C-18 fatty acids. Following upward transition there was a 2.5 fold increase in the proportion of 18:1 after 48h, accompanied by a progressive decrease in the proportion of 18:3 to reach a level after 48h 26% below that found in cells grown at 15°; a concomitant decrease in 16:0 level from 7.7% at 15° to 6.0% at 28° was also apparent (Table 4.10).

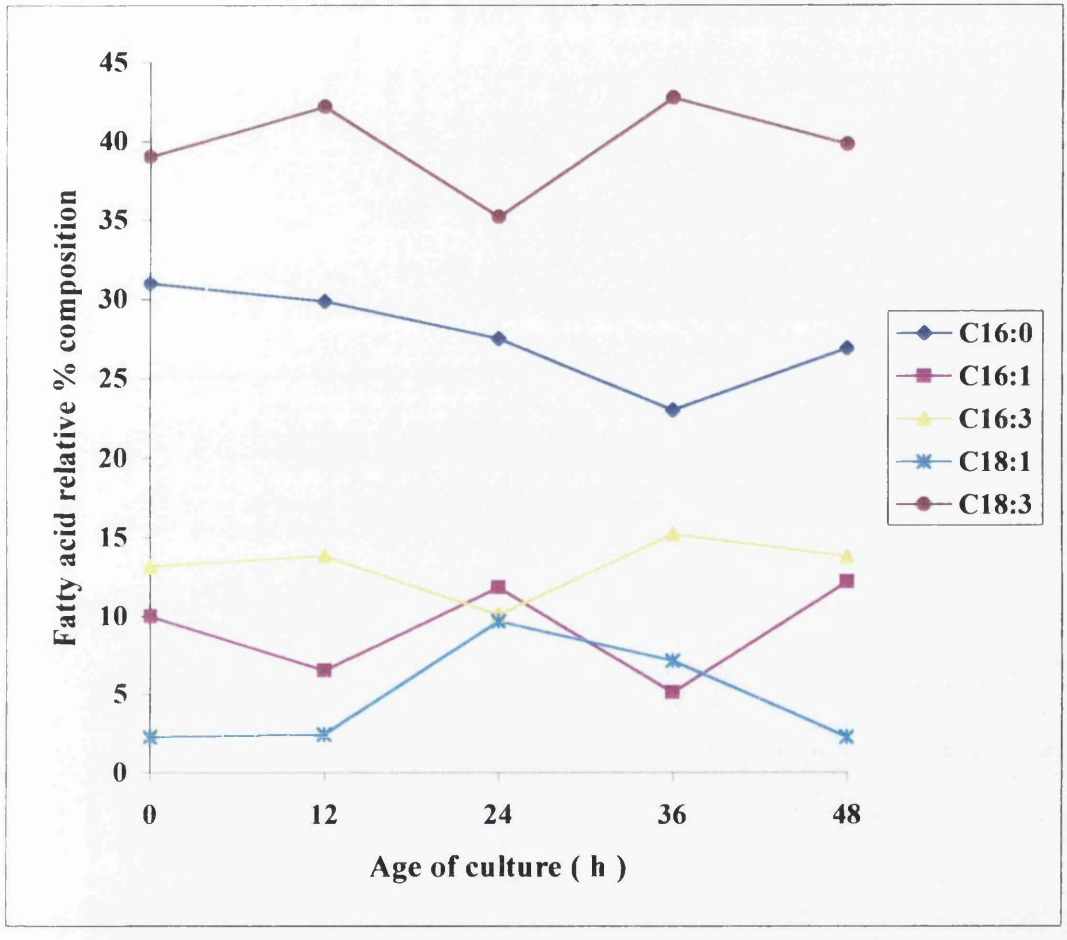


Fig 4.8 Relative % composition of the major fatty acids in the DGDG fraction from *Aphanizomenon* sp. culture following a temperature shift from 15° to 28°.

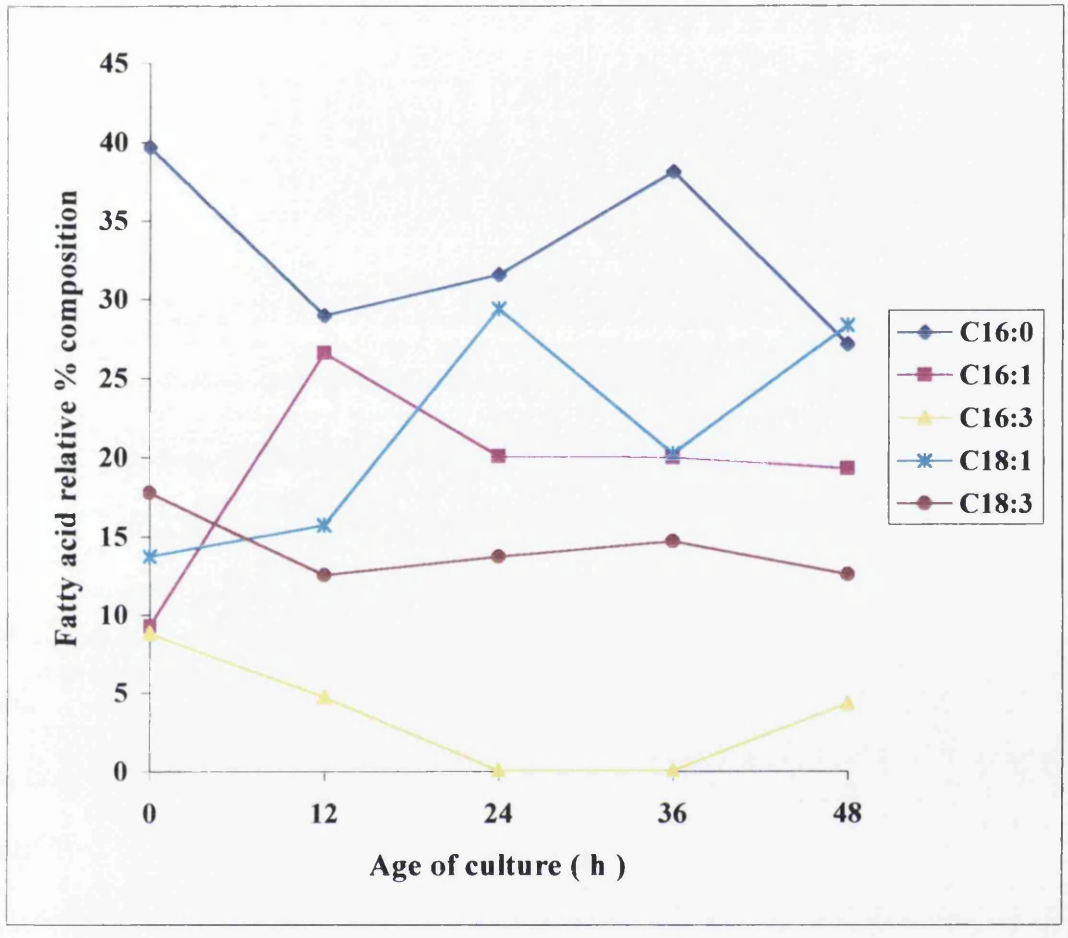


Fig 4.9 Relative % composition of the major fatty acids in the PG fraction from *Aphanizomenon* sp. culture following a temperature shift from 15° to 28°.

4.4.5 Sulphoquinovosyldiacylglycerol

The fatty acid profile in SL from cells grown at 15° was quite distinct from that of the other three glycerolipids, due to the high proportion of 16:0 present (Fig. 4.10) which appeared to remain relatively constant following the temperature transition. Following the temperature shift a 2.8 fold increase in the proportion of 16:1 which remained a relatively minor component was observed. No 16:2 could be detected in SL after growth at 15° and only a small amount of 16:3 could be found, and the level of this fatty acid did not alter significantly following the temperature transition. On the other hand, raising the culture temperature had significant effects on the proportions of 18:1 and 18:3 in SL. The proportion of 18:1 increased 3.9 fold reaching a maximum (17% of SL fatty acid) within 24h of transition to 28°, whilst over the same time period a substantial decrease in the level of 18:3 was observed within 48h from 32% to 20% following the temperature shift. Thus the pattern in SL was broadly in agreement with that seen in the C-18 fatty acid pool in the total lipid fraction.

4.5 Discussion

The temperature shift from 15° to 28° caused an increase in the level of saturation in the fatty acyl chains of the membrane glycerolipids. Among C-16 acids, there was no evidence for an increase in the proportion of 16:0, but a decrease in 16:3 and a marked increase in 16:1 were observed (Table 4.6). Also, changes in C-18 fatty acids were observed. In the total lipid, a small decrease in 18:3 was found, accompanied by an approximate doubling in the proportion of 18:1 within 48h of the upward temperature shift and the level of 16:3 also decreased whilst 16:1 increased

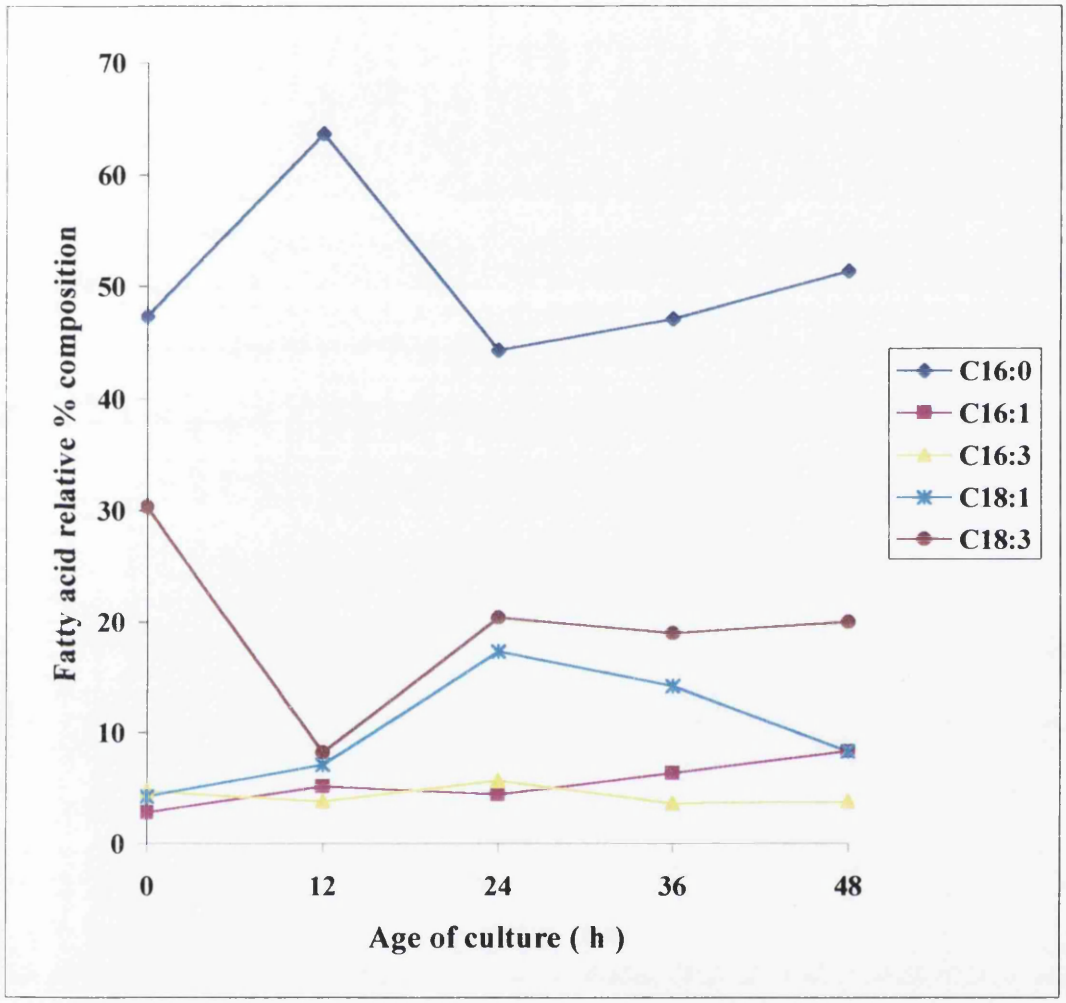


Fig 4.10 Relative % composition of the major fatty acids in the SL fraction from *Aphanizomenon* sp. culture following a temperature shift from 15° to 28°.

Table 4.6 Changes in fatty acid relative % composition of lipid fractions from *Aphanizomenon* sp following temperature transition from 15° to 28° for 48h.

Fatty acid increase (↑) or decrease (↓)

Lipid fraction	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3
Total lipid	-	↑↑↑	-	↓↓	↑	↑↑↑	↑↑↑	↓
MGDG	-	↑↑↑	-	↓↓	-	↑↑↑	↑↑↑	↓
DGDG	↓	↑↑	-	-	↑↑↑	-	-	-
PG	↓↓	↑↑↑	nd	nd	↓↓	↑↑↑	-	↓↓
SL	-	↑↑↑	nd	-	↑↑↑	↑↑↑	↑↑↑	↓↓↓

↑↑↑	(≤ 30%)	↓	(≤ 10% decrease)
↑↑	(≤ 20%)	↓↓	(≤ 20% decrease)
↑	≤ 10%	↓↓↓	(≤ 30% decrease)
-	= 0 - 10		
nd	Not detected		

significantly (Table 4.6). The amount of the saturated C-16 and C-18 fatty acids however remained constant during temperature adaptation. These findings suggest that the observed changes in fatty acid composition are most probably due to accelerated syntheses of 16:1 and 18:1 and a substitution of the pre-existing polysaturated fatty acids by newly synthesised fatty acids.

Similar changes were obtained in MGDG following the temperature shift from 15 ° to 28° (Table 4.6). The saturated fatty acids remained unchanged whilst the polyunsaturated fatty acid decreased and the less unsaturated fatty acids, 16:1 and 18:1, increased. The similarity in the fatty acid-patterns and changes observed during temperature adaptation in the two galactolipid fractions was consistent with the conversion of MGDG to DGDG by galactosylation during the acclimation to higher temperature, as has been suggested previously (Sato and Murata, 1980a). The acidic lipid SL contained a substantial proportion of 16:0 which remained unchanged following the upward temperature shift. Moreover, the trend of the fatty acids present in PG was similar to that in SL. The responses seen in the glycoglycerolipid C-16 fatty acids of *Aphanizomenon* sp are different from those reported by Sato and Murata (1980a), who found that in the first 5h after a temperature shift from 22° to 38° in *A. variabilis*, the relative content of 16:0 increased whilst 16:1 decreased specifically in MGDG and DGDG. Also in *A. variabilis*, a decrease in the proportion of 18:3 was observed in all of the lipid classes which was similar to that found in *Aphanizomenon* sp. The results observed during the temperature shift from 15° to 28° concluded that there is a decrease in the level of unsaturation similar to that found in other cyanobacterial species, but that the increase in saturation level may be brought about by different mechanisms from those observed previously.

CHAPTER FIVE

Studies on The Stereospecific Distribution of Fatty Acids in *Aphanizomenon* sp. Glycerolipids

5.1 Introduction

The studies described earlier in Chapter 3 and 4 established that *Aphanizomenon* sp. modulates the fatty acid composition of its membrane glycerolipids in response to changes in its growth temperature. This chapter describes further studies into the detail of these changes by an analysis of the stereospecific distribution of fatty acids (see chapter 1.3.1) within each of the major glycerolipid classes from *Aphanizomenon* sp. cultures grown either isothermally at 28° or at 15° and during temperature transition from 28° to 15°.

This was determined by use of *Rhizopus* lipase (Sato and Murata, 1988), which specifically hydrolyses fatty acyl substituents at the *sn*-1 position of glycerolipids (Fisher *et al.*, 1973; Sato and Murata, 1980, 1982) to generate the corresponding lyso-glycerolipid in which the *sn*-2 fatty acyl substituent is retained. Subsequent transesterification of the fatty acid released by *Rhizopus* lipase and the lysoglycerolipid produced yields the fatty acid methyl esters of the fatty acids originally at *sn*-1 and *sn*-2 respectively, which can be compared with the 'overall' fatty acid composition obtained by transesterification of the original glycerolipid (Fisher *et al.*, 1973). More reliable results are obtained by use of the *sn*-2 fatty acyl composition in combination with the original glycerolipid fatty acid composition to determine the composition of the *sn*-1 fatty acid content by difference (Christie ,1982).

In this Chapter, the positional distribution of fatty acids present in the individual lipid classes from *Aphanizomenon* sp. cultured isothermally at two different temperatures, either 'high temperature' (28°) or 'low temperature' (15°), and during a temperature transition from 28° to 15° was investigated. Cells of *Aphanizomenon sp.* cultures grown at either 28° or at 15° or during the temperature

transition were harvested and the total lipid fraction extracted according to the methods described in Chapter 2. The major glycerolipid classes were separated by preparative silica gel-G TLC (Chapter 2.5), and samples subjected to hydrolysis with *Rhizopus* lipase as described in Chapter 2.12 for the appropriate time (see 5.1a below). The lysoglycerolipid containing the *sn*-2 fatty acid was recovered by double-development TLC (Chapter 2.12.2). Samples of each lysoglycerolipid and the original glycerolipid from which the sample was derived were individually transesterified (see chapter 2.6) and the resulting FAME fractions subjected to GLC analysis as described earlier in Chapter 2.8.

5.1.1 Optimisation of Lipase Hydrolysis Times.

Glycerolipid fractions were recovered from preparative-TLC plates by the method described earlier in Chapter 2.7.2(a). Each lipid class was dissolved in $CHCl_3/CH_3OH$ (2:1 v/v) and incubated with lipase under the conditions described in Chapter 2.12.1 for periods between 10-60 minutes (Sato and Murata, 1988), after which the incubation was terminated. The products were subjected to double-development TLC analysis, and the relative intensity of the lysoglycerolipid band after spraying with primulin and visualisation under uv light was assessed, and the optimal hydrolysis times shown in Table 2.1 established.

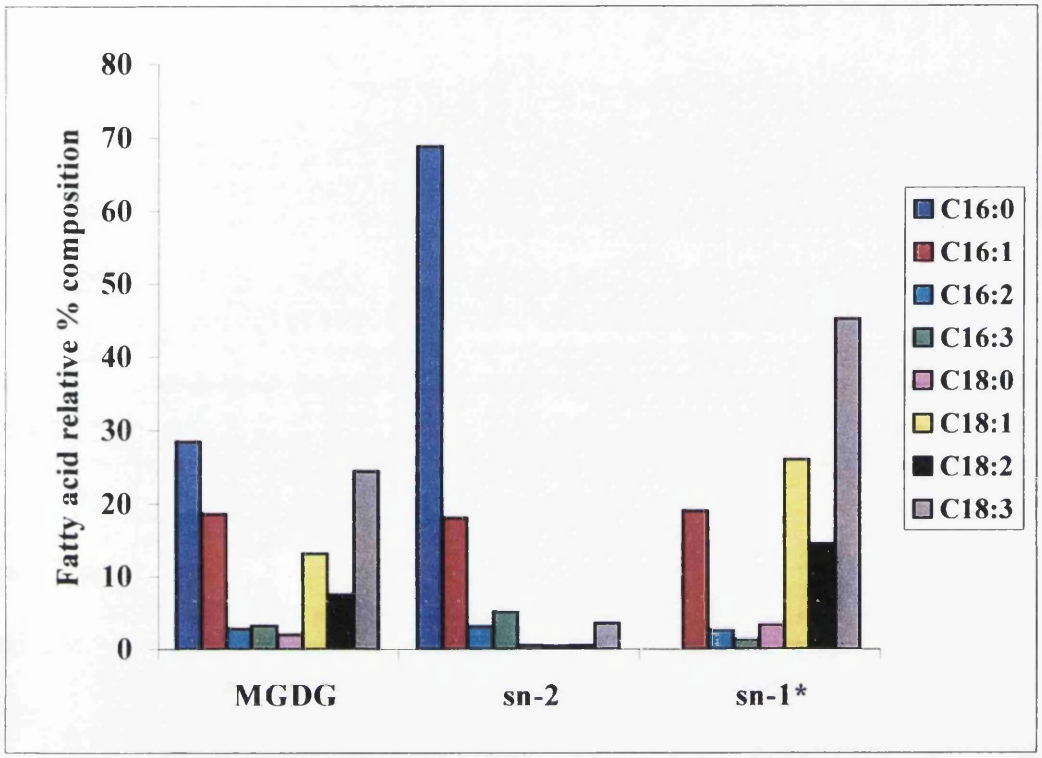


Fig. 5.1 The positional distribution of fatty acids in MGDG isolated from Aphanizomenon sp. cultured at 28°

*** Values for fatty acid composition of less than 0 obtained by difference are shown as 0 on the figure**

5.2 Stereospecific Analysis of Fatty Acid Distribution in Glycerolipids from *Aphanizomenon* sp. cultures grown at 28°.

5.2.1 Monogalactosyldiacylglycerol

Fig. 5.1 shows the fatty acid composition of the MGDG fraction together with the positional distribution at *sn*-1 and *sn*-2. In the MGDG fraction the sum of C-16 acids was about 60% with C-18 acids constituting about 40%. At the *sn*-2 position fatty acids with a C-16 chain represented 95% of the fatty acid, whilst at the *sn*-1 position about 90% of the fatty acid was of C-18 chain length (Table 5.1, Appendix 4).

The observed fatty acid distribution is in general agreement with that reported for other Group 2 cyanobacteria in which C-16 fatty acids are esterified exclusively at *sn*-2 whilst the C-18 fatty acids predominate at *sn*-1 (Sato and Murata, 1982; Murata *et al.*, 1992). However, the *sn*-2 position in *Aphanizomenon* sp. MGDG was found to contain about 5% of C-18 fatty acids rather than being totally esterified with C-16 fatty acids as reported for *Synechoccus* PCC 7002 (Murata *et al.*, 1992) and *A. variablis* (Sato and Murata, 1982), and a significant proportion (23%) of unsaturated C-16 fatty acids were found at *sn*-1 (Fig 5.1)

5.2.2 Digalactosyldiacylglycerol

Fig 5.2 shows the fatty acid composition of the DGDG fraction together with the positional distribution of fatty acids at *sn*-1 and *sn*-2. In the DGDG fraction there was an approximately equal amount of C- 16 and C- 18 chain length fatty acid (Table 5.2, Appendix 4). At the *sn*-2 position fatty acids with a C-16 chain represented 92% of the fatty acid, whilst at the *sn*-1 position about 90% of the fatty acid was of C-18 chain length.

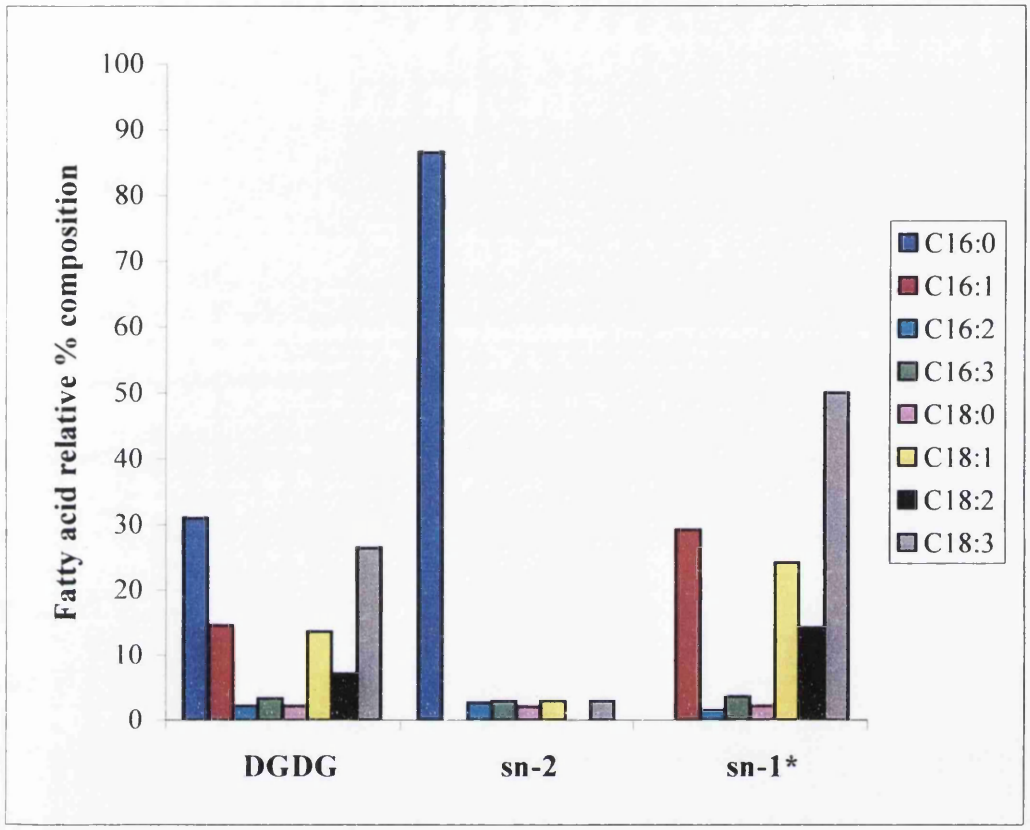


Fig. 5.2 The positional distribution of fatty acids in DGDG isolated from Aphanizomenon sp. cultured at 28°

*** Values for fatty acid composition of less than 0 obtained by difference are shown as 0 on the figure**

The observed fatty acid distribution is in general agreement with that reported by Murata *et al.,* 1992 in that C-16 fatty acids are esterified exclusively at *sn*-2 whilst the C-18 fatty acids predominate at *sn*-1. In a similar manner to MGDG, the *sn*-2 position of DGDG in *Aphanizomenon* sp. was found to contain about 8% of C-18 fatty acids rather than being totally esterified with C-16 fatty acids.

5.2.3 Phosphatidylglycerol

Fig 5.3 shows the fatty acid composition of the PG fraction together with the positional distribution at *sn*-1 and *sn*-2. In the PG fraction the sum of C-16 acids was about 45% and no polyunsaturated C-16 fatty acids were detected, whilst C-18 fatty acids represented some 55% of the total fatty acid (Table 5.3 Appendix 4). At the *sn*-2 fatty acids with a C-16 chain represented about 72%, whilst at the *sn*-1 position about 94% of the fatty acid was the unsaturated C-18 chain length. At the same time it was found that at the *sn*-1 position about 27% of the saturated C-16 acids was esterified, and that 18:0 was esterified exclusively at the *sn*-2 position.

5.2.4 Sulphoquinovosyldiacylglycerol

Fig. 5.4 shows the fatty acid composition of SL fraction together with the positional distribution at *sn*-1 and *sn*-2. In the SL fraction the percentage of C-16 acids was about 63% and was entirely composed of saturated fatty acid (16:0) whilst the C-18 fatty acids (total 37%) contained saturated, monoenoic and polyunsaturated components (Table 5.4, Appendix 4). At *sn*-2, 16:0 represented about 94% of the fatty acid, whilst at the *sn*-1 position some 69% of the fatty acid was of C-18 chain length whilst there was about 31% of C-16 fatty acid esterified at *sn*-1.

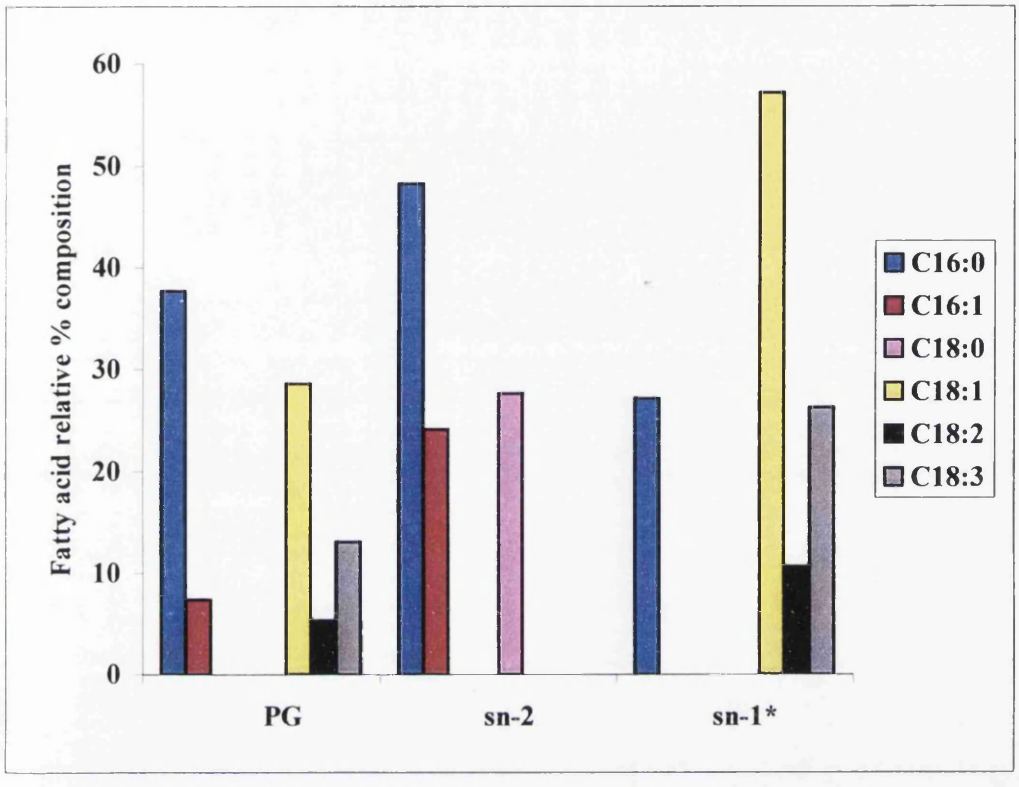


Fig. 5.3 The positional distribution of fatty acids in PG isolated from Aphanizomenon sp. cultured at 28°

*** Values for fatty acid composition of less than 0 obtained by difference are shown as 0 on the figure**

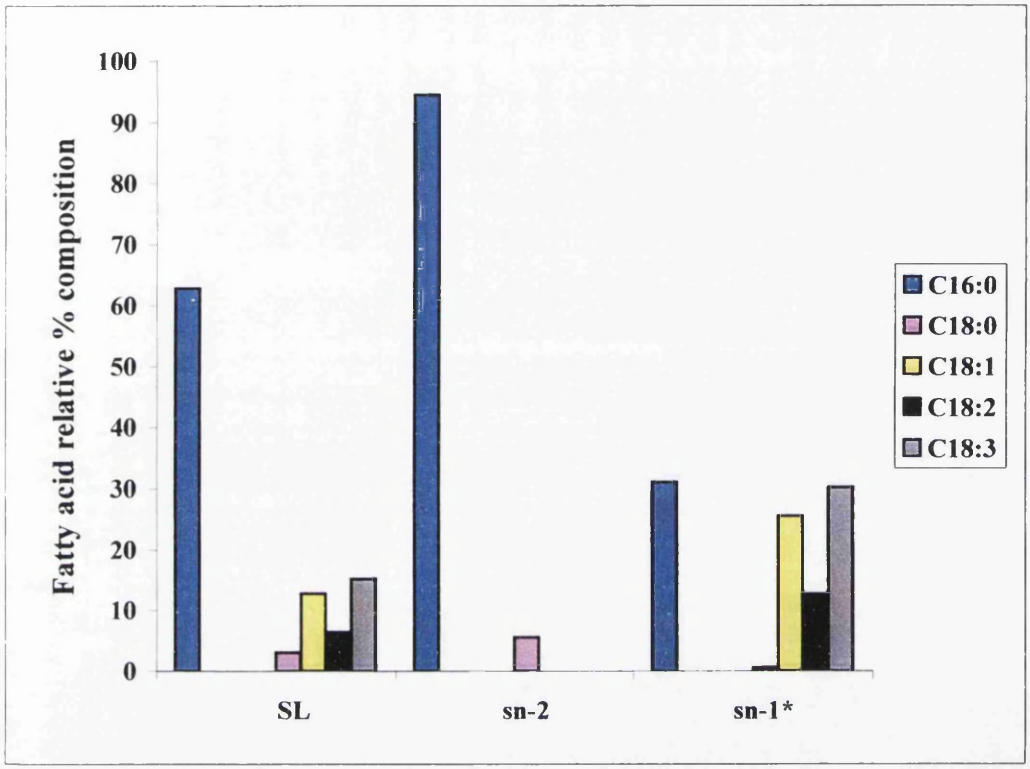


Fig. 5.4 The positional distribution of fatty acids in SL isolated from Aphanizomenon sp. cultured at 28°

*** Values for fatty acid composition of less than 0 obtained by difference are shown as 0 on the figure**

5.3 Stereospecific Analysis of Fatty Acid Distribution in Glycerolipids from *Aphanizomenon* sp. Cultures Grown at 15°

5.3.1 Monogalactosyldiacylglycerol

Fig. 5.5 show the fatty acid composition together with the positional distribution at *sn*-1 and *sn*-2 of the MGDG fraction from *Aphanizomenon* sp cultured at low temperature (15°). In the MGDG fraction the sum of C-16 and C-18 fatty acids was about equal (Table 5.5 Appendix 4). At *sn*-2 fatty acids with a C-16 chain represented 95% of the total fatty acid, whilst at the *sn*-1 position about 98% of the fatty acid was of C-18 chain length. The observed distribution of fatty acid chain lengths in MGDG at low temperature follows a similar pattern to that observed at high growth temperature described above, and is in agreement with that reported for other Group 2 cyanobacteria in which C-16 fatty acids are esterified exclusively at *sn*-2 whilst the C-18 fatty acids predominate at *sn*-1 (Sato and Murata, 1982; Murata *et al.*, 1992). A notable feature in *Aphanizomenon* however was the high proportion of 16:3 (35%) located almost exclusively at *sn*-2.

5.3.2 Digalactosyldiacylglycerol

Fig. 5.6 shows the fatty acid composition and positional distribution at *sn*-1 and *sn*-2 of the DGDG fraction from *Aphanizomenon* sp. culture at low temperature (15°). In the DGDG fraction the sum of C-16 and C-18 fatty acids was approximately equal (Table 5.6 Appendix 4). At the *sn*-2 position fatty acids with a C-16 chain represented 95% of the fatty acid, whereas at the *sn*-1 position about 93% of the fatty acid was of C-18 chain length. It was clear that the *sn*-2 position was esterified almost totally by C-16 fatty acids amongst which 16:3 was a prominent

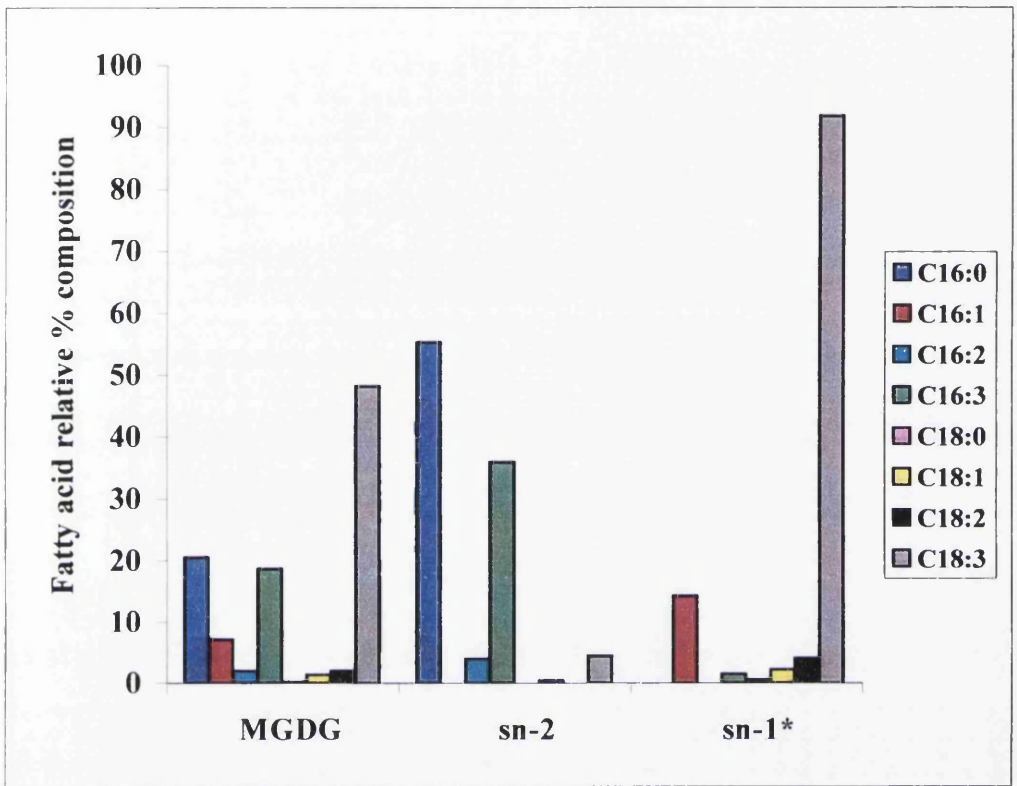


Fig. 5.5 The positional distribution of fatty acids in MGDG isolated from *Aphanizomenon* sp. cultured at 15°

*** Values for fatty acid composition of less than 0 obtained by difference are shown as 0 on the figure**

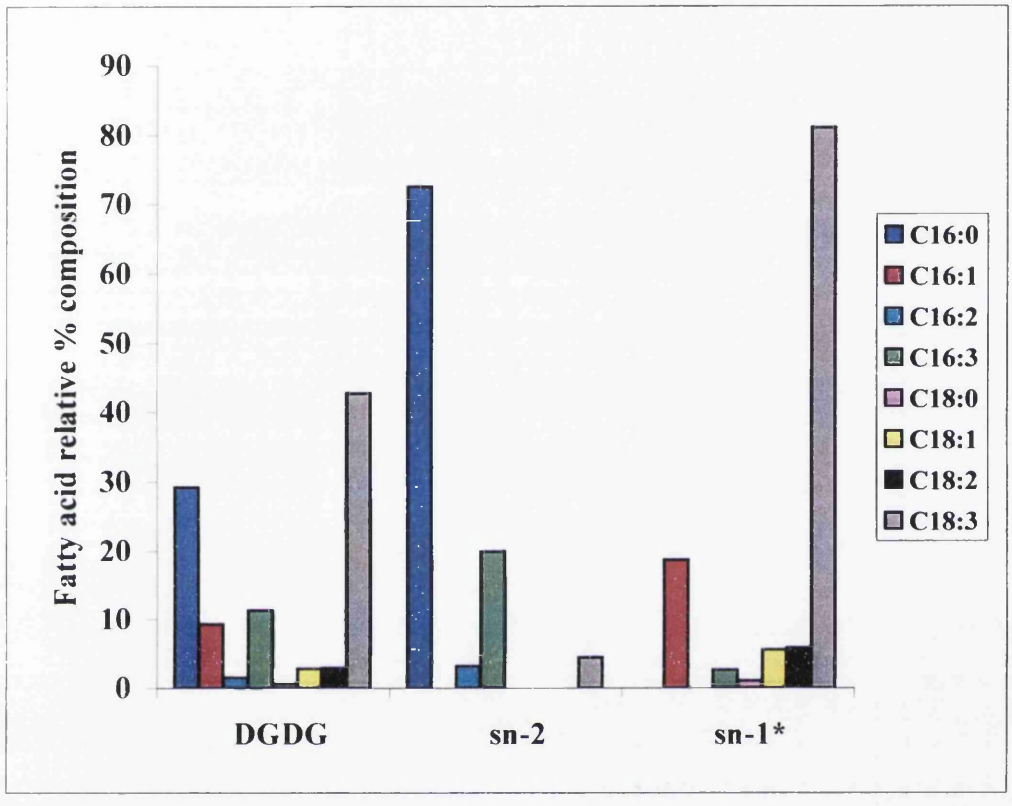


Fig. 5.6 The positional distribution of fatty acids in DGDG isolated from *Aphanizomenon* sp. cultured at 15°

*** Values for fatty acid composition of less than 0 obtained by difference are shown as 0 on the figure**

component whilst the *sn*-1 position was esterified predominantly by C-18 fatty acids.

5.3.3 Phosphatidylglycerol

Fig. 5.7 shows that the fatty acid composition of the PG fraction together with the positional distribution of fatty acids at *sn*-1 and *sn*-2 at 15°. In the PG fraction the sum of C-16 and C-18 fatty acids was approximately equal (Table 5.7 Appendix 4). At *sn*-2 fatty acids with a C-16 chain length represented about 88% of the total, whilst at the *sn*-1 position C-18 fatty acids predominated, but a substantial proportion (30%) of monoenoic C-16 fatty acid was found.

5.3.4 Sulphoquinovosyldiacylglycerol

Fig. 5.8 shows the fatty acid composition of SL fraction together with their positional distribution at *sn*-1 and *sn*-2 at 15°. In the SL fraction the C-16 acids was entirely accounted for by 16:0 and it represented about 60% of the fatty acids in SL (Table 5.8 Appendix 4). The *sn*-2 position was excusively esterified with 16:0, whilst C-18 fatty acids predominated at *sn*-1.

5.4 Discussion

The results obtained in the studies described above confirmed that the growth temperature affected the relative proportions of individual fatty acids and the degree of unsaturation in the fatty acyl chains of the glycerolipids. The extent of unsaturation is enhanced at low temperature within the range of physiological growth conditions. The positional distribution of fatty acids in all lipid classes from cultures grown at 28° or 15° was characterised by the predominance of C-18 fatty acids at the

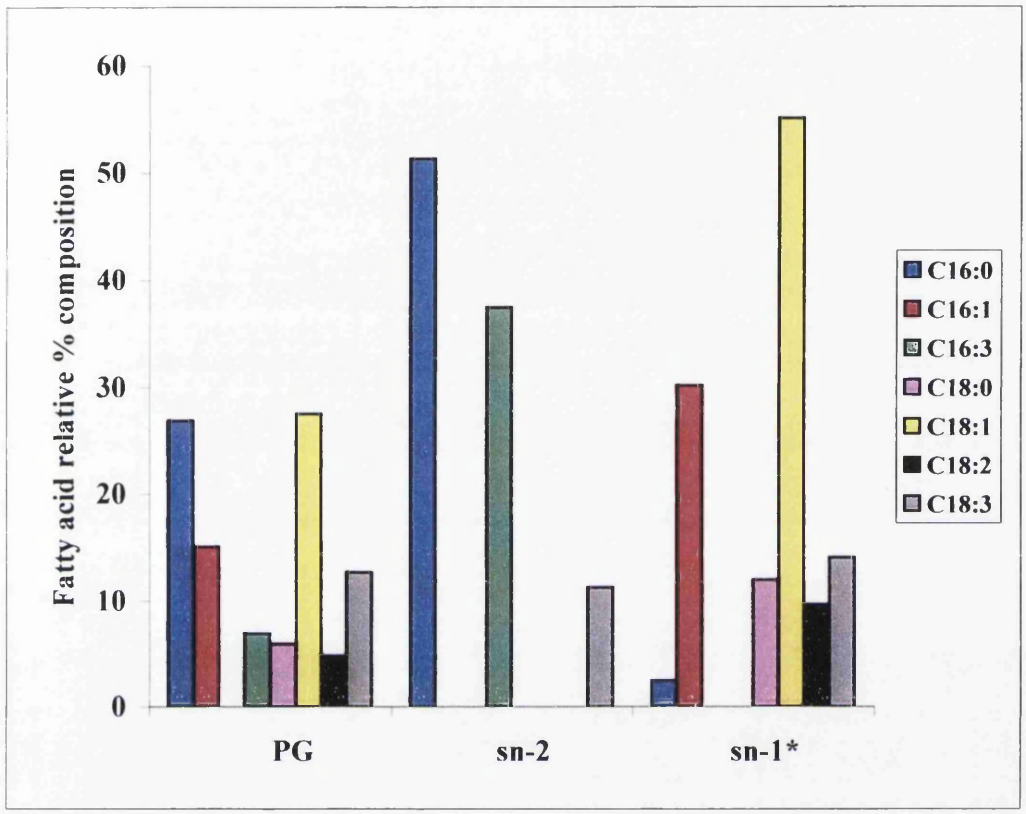


Fig. 5.7 The positional distribution of fatty acids in PG isolated from *Aphanizomenon* sp. cultured at 15°

*** Values for fatty acid composition of less than 0 obtained by difference are shown as 0 on the figure**

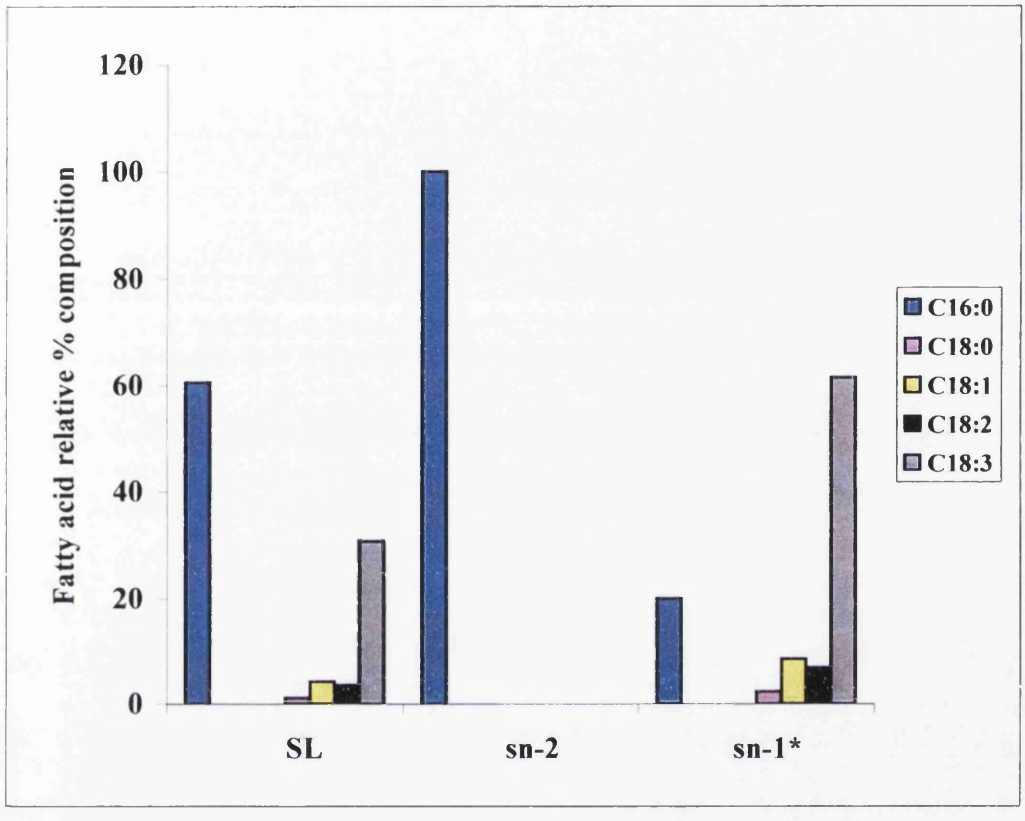


Fig. 5.8 The positional distribution of fatty acids in SL isolated from *Aphanizomenon* sp. cultured at 15°

*** Values for fatty acid composition of less than 0 obtained by differendence are shown as 0 on the figure**

sn-1 position and C-16 fatty acids at the *sn*-2 position, in a pattern broadly consistent with that described for other Group 2 cyanobacteria (Sato and Murata 1979). When *Aphanizomenon* sp. cells grown at low temperature (15°) are compared with cells grown at high temperature (28°) several differences in fatty acid composition could be detected. Low growth temperature led to an increase in the proportion of unsaturated fatty acid and to a decrease in the proportion of saturated fatty acids in all the lipid classes. In MGDG and DGDG fractions, the relative proportion of 16:0 at the *sn*-2 position in cells grown at 28° was 68.8% and 86.5%, respectively whilst it reduced to 55.3% and 72.6% at 15° in MGDG and DGDG (Tables 5.1 and 5.2, Appendix 4). This reduction in 16:0 was accompanied by a sharp increase in the proportion of 16:3 at *sn*-2 of the MGDG and DGDG fractions in cells grown at 15°. At the lower temperature an approximate doubling in the relative proportion of 18:3 fatty acid at *sn*-1 position in MGDG and DGDG was also observed, whilst the relative proportions of 18:1 and 18:2 at *sn*-1 were decreased. In the acidic glycerolipids, PG and SL, the relative proportions of fatty acids were also affected. The 16:0 fatty acid in PG fraction was esterified mainly at *sn*-2 position in cells grown at 28°, where it represented 48.2% of the fatty acid, and was little changed (51.3%) in cells grown at 15° (Tables 5.3 and 5.7, Appendix 4). At *sn*-2 16:1 represented 24.1% of fatty acid at 28° , but this fatty acid could not be detected at 15° , whilst 16:3, which was absent at 28°, constituted 37.3% of the PG *sn*-2 fatty acid at 15°. At the lower temperature 16:1 was also a prominent component at *sn*-1. The C-18 fatty acid composition at the *sn*-1 position was also affected by temperature. When cells were grown at 15°, the relative proportion of 18:1 at *sn*-1 in MGDG, DGDG, and SL decreased, whilst the 18:3 increased. In contrast, at *sn*-1 position in PG the relative proportion of 18:1 increased and 18:3 decreased. In the SL fraction,

16:0 was the only C-16 fatty acid at the *sn*-2 position when cells were cultured at either high temperature (28°) or low temperature (15°), and there was little difference in the relative proportion present at the two growth temperatures (Tables 5.4 and 5.8, Appendix 4). C-18 acids were located almost entirely at the *sn*-1 position in SL from cells grown at 28° and 15°, and a very substantial increase in the relative proportion of 18:3 fatty acid at *sn*-1 was seen in cells grown at 15°. This doubling of the 18:3 content was accompanied by marked decreases in the relative proportion of 18:1 and 18:2 observed at 28°. In spite of all these changes in fatty acid relative proportion, the positional distribution was not altered except that in PG fraction the 16:1 fatty acid was esterified in *sn*-1 position instead of *sn*-2 position in cells grown at 15°. This finding is in contrast to that of Sato *et al.*, 1979. These workers found that in *Anabaena variabilis* the C-18 and C-16 acid were located almost exclusively at *sn*-1 and *sn*-2 position respectively in all the lipid classes. It thus appears that in group 2 cyanobacterium *Aphanizomenon* sp, as in *A. variabilis*, adaptation of fatty acid composition is different from that in Group 1 cyanobacteria e.g. *Anacystis nidulans*. In this organism Sato *et al.*, (1979), found that in growth at reduced temperature the change in chain length was found at *sn*-1 position and the change in unsaturation was at *sn*-2 position; the monounsaturated acids were dominant at *sn*-1 and saturated ones at *sn*-2. When the growth temperature was changed, the chain length of monounsaturated acids varied at *sn*-1, and the desaturation of C-16 acids in galactolipids was influenced at *sn*-2. In the acidic lipids (SL and PG), only palmitic acid was esterified to *sn*-2, and no change with growth temperature was observed at this position.

5.5 Stereospecific Analysis of Fatty Acid Distribution in Glycerolipids from *Aphanizomenon* sp. Cultures During Temperature Transition

Changes in fatty acids distribution during the temperature transition from 28° to 15° were studied in this part of work. The conditions for these experiments were established during the temperature adaptation studies described earlier, (Chapter 2.11), and enzymatic hydrolysis using *Rhizopus* lipase was performed as described above.

5.5.1 Monogalactosyldiacylglycerol

. Table 5.9 shows the fatty acid composition of MGDG fraction together with the positional distribution at *sn*-1 and *sn*-2 during the temperature shift from 28° to 15°. In the MGDG fraction at 0h in 28° the sum of C-16 acids was about 52.5% and fatty acids with a C-16 chain represented 93% of the *sn*-2 fatty acid, whilst at *sn*-1 position about 88% of fatty acid was of C-18 chain length. After 24h from temperature shift to 15° the sum of C-16 fatty acids in MGDG fraction was not significantly changed (total of 50%) and the C-16 chain length represented 92.3% whilst at the *sn*-1 position about 91.3% of the *sn*-2 fatty acids of fatty acid was of C-18 chain length. Within 48h from temperature shift to 15°, the sum of C-16 acids in MGDG fraction was 47.1%, and there was no significant change in either the proportion of *sn*-2 fatty acid with C-16 chain, or *sn*-1 fatty acid of C-18 chain length. At the *sn*-2 position, 16:0 decreased from 65% to 60.9% within 48h, whilst in the same time period 16:2 and 16:3 increased from 2.8% to 5.1% and from 5.1% to 7.1% respectively, and at 15° at the *sn*-1 position after 48h there was a decrease in the relative proportion of 18:1 from 26.6% to 22.4% and an increase in the proportion of 18:3 from 48.1% to 58.3%.

Table 5.9 Stereospecific analysis of fatty acid distribution in *Aphanizomenon* sp in galactolipids during temperature shift from 28° to 15°.

	MGDG 0h				MGDG 24h				MGDG 48h			
Fatty acids	TL %	Overall%	sn - 2 %	*sn - 1 %	TL %	Overall%	sn - 2 %	*sn - 1 %	TL %	Overall%	sn - 2 %	*sn - 1 %
C16:0	37.2	27.0	65.0	-11.0	34.3	24.0	70.0	-22.0	27.0	19.0	60.9	-22.9
C16:1	15.7	19.4	20.8	18.0	15.5	18.7	11.4	26.1	19.7	21.5	21.4	21.6
C16:2	3.1	2.8	2.8	2.9	1.9	3.2	4.2	2.3	3.0	4.0	5.1	3.0
C16:3	1.5	3.3	5.1	1.4	3.1	4.5	6.8	2.3	5.0	5.5	7.1	4.0
C18:0	1.1	0.6	nd	1.3	1.7	0.5	nd	1.0	1.0	0.4	nd	0.9
C18:1	11.6	14.2	1.8	26.6	9.2	9.8	2.0	17.6	6.8	11.2	nd	22.4
C18:2	6.5	6.4	nd	12.8	7.1	6.7	nd	13.4	6.9	6.3	nd	12.7
C18:3	23.4	26.3	4.5	48.1	27.3	32.5	5.7	59.4	31.9	31.9	5.6	58.3

	DGDG 0h				DGDG 24h				DGDG 48h			
Fatty acids	TL %	Overall%	sn - 2 %	sn - 1 %	TL %	Overall%	sn - 2 %	sn - 1 %	TL %	Overall%	sn - 2 %	*sn - 1 %
C16:0	37.2	38.9	97.9	-20.0	34.3	29.8	97.5	-38.0	27.0	26.4	100.0	-47.3
C16:1	15.7	12.6	nd	25.2	15.5	14.6	1.0	28.3	19.7	14.1	nd	28.3
C16:2	3.1	2.1	nd	4.2	1.9	2.5	nd	nd	3.0	2.6	nd	5.3
C16:3	1.5	3.3	nd	5.1	3.1	3.7	nd	nd	5.0	3.8	nd	7.6
C18:0	1.1	2.1	2.1	2.1	1.7	1.3	1.5	1.1	1.0	1.2	nd	2.4
C18:1	11.6	13.5	nd	27.1	9.2	18.4	nd	36.9	6.8	21.0	nd	41.9
C18:2	6.5	7.1	nd	14.2	7.1	7.8	nd	15.6	6.9	8.1	nd	16.1
C18:3	23.4	20.4	nd	40.8	27.3	21.9	nd	43.8	31.9	23.6	nd	47.1

*** calculated by difference**

5.5.2 Digalactosyldiacylgylcerol

Table 5.9 shows the fatty acid composition of DGDG fraction together with the positional distribution of *sn*-1 and *sn*-2 during the temperature shift from 28° to 15°. In the DGDG fraction in 0h at 28° the sum of C-16 acid represented 56.9% and fatty acid with a C-16 chain which was mainly 16:0 represented 97.9% of fatty acid at the *sn*-2 position, whilst the *sn*-1 position contained about 84.1% of C-18 acid. After 24h at 15° the sum of C-16 acids in the DGDG fraction decreased to 50.6%, whilst the relative proportion of 16:0 at sn-2 was relatively unchanged and represented 97.5% of the fatty acid esterified this position. However at *sn*-1, the sum of C-18 acid increased significantly from 84.1% to 97.1%. Within 48h the sum of C-16 acids in DGDG declined to 47%, and the *sn*-2 position was completely esterified by 16:0, whilst at the *sn*-1 position the relative proportion of C-18 acids showed a small increase. 18:2 increased from 14.16% to 16.12% and 18:3 increased from 40.84% to 47.12%, whilst there was a 35.40% increase in 18:1 during that period.

5.5.3 Phosphatidylglycerol

Table 5.10 shows the fatty acid composition of PG fraction together with the positional distribution at *sn*-1 and *sn*-2 during the temperature shift from 28° to 15°. In the PG fraction from the culture grown at 28° the sum of C-16 fatty acids was about 53% and fatty acids with a C-16 chain represented about 88% of the fatty acid at *sn*-2, whilst at the *sn*-1 position about 82% of the fatty acid was 18:1, the balance being composed of 16:0. After 24h from temperature shift to 15°, the sum of C-16 acid in the PG fraction was decreased to 38.0%, and at *sn*-2 the proportion of C-16 fatty acids was not significantly changed and represented 90.9% of the total, whilst at the *sn*-1 position an increase in C-18 fatty acids was apparent. After 48h at 15° the

Table 5.10 Stereospecific analysis of fatty acid distribution in *Aphanizomenon* sp. in acidic lipids during temperature shift from 28° to 15°

	PG 0h				PG 24h				PG 48h			
Fatty acids	TL %	Overall%	sn - 2 %	*sn - 1 %	TL %	Overall%	sn - 2 %	*sn - 1 %	TL %	Overall%	sn - 2 %	*sn - 1 %
C16:0	37.2	48.1	65.1	31.0	34.3	27.4	72.0	-17.3	27.0	24.8	61.3	-11.7
C16:1	15.7	5.1	23.3	-13.1	15.5	10.6	19.0	2.3	19.7	11.0	10.9	11.1
C16:2	3.1	nd	nd	nd	1.9	nd	nd	nd	3.0	nd	nd	nd
C16:3	1.5	nd	nd	nd	3.1	nd	nd	nd	5.0	nd	nd	nd
C18:0	1.1	5.5	11.6	-0.6	1.7	7.8	9.4	6.3	1.0	8.6	nd	17.2
C18:1	11.6	41.4	nd	82.7	9.2	47.7	nd	95.4	6.8	47.7	27.8	67.6
C18:2	6.5	nd	nd	nd	7.1	6.5	nd	13.0	6.9	7.9	nd	15.7
C18:3	23.4	nd	nd	nd	27.3	nd	nd	nd	31.9	nd	nd	nd

	SL 0h				SL 24h				SL 48h			
Fatty acids	TL %	Overall%	sn - 2 %	sn - 1 %	TL %	Overall%	sn - 2 %	sn - 1 %	TL %	Overall%	sn - 2 %	sn - 1 %
C16:0	37.2	46.5	96.8	-3.8	34.3	51.8	96.8	6.7	27.0	48.0	95.6	0.5
C16:1	15.7	13.8	nd	27.7	15.5	10.8	nd	21.6	19.7	6.0	nd	12.0
C16:2	3.1	nd	nd	nd	1.9	nd	nd	nd	3.0	nd	nd	nd
C16:3	1.5	nd	nd	nd	3.1	nd	nd	nd	5.0	nd	nd	nd
C18:0	1.1	3.3	3.2	3.4	1.7	2.1	3.2	1.0	1.0	4.1	4.4	3.7
C18:1	11.6	27.4	nd	54.7	9.2	30.5	nd	61.0	6.8	37.4	nd	74.8
C18:2	6.5	4.9	nd	9.8	7.1	3.6	nd	7.3	6.9	3.8	nd	7.5
C18:3	23.4	4.1	nd	8.2	27.3	1.2	nd	2.5	31.9	nd	nd	nd

*** calculated by difference**

sum of C-16 acids in the PG fraction was largely unchanged (35.8%) and at *sn*-2 the C-16 chain fatty acids had decreased to 72.2% and there was a concomitant appearance of 18:1. At the *sn*-1 position the sum of C-18 acid was about 94%, similar to the level observed after 24h. The PG fraction showed a characteristically different response from those of the galactolipid and SL fraction, in that after 48h at reduced temperature, a substantial proportion (27%) of 18:1 was at the *sn*-2 position of the phospholipid, whereas in each of the other lipid classes the *sn*-2 position contained at most only constant minor amount of C-18 fatty acid (Table 5.10).

5.5.4 Sulphoquinovosyldiacylglycerol

Table 5.10 shows the fatty acid composition of SL fraction together with the positional distribution at *sn*-1 and *sn*-2 during the temperature shift from 28° to 15°. At 0h the percentage of C-16 chain length fatty acid in the SL fraction was about 60%, and 16:0 represented about 96.8% of the fatty acid at *sn*-2, whilst at *sn*-1 position about 76.2% of the fatty acid was of C-18 acids, of which 18:1 (54.7%) was the most substantial component. After 24h the C-16 fatty acid content of SL was 62.6% and 16:0 represented 96.8% of the *sn*-2 fatty acids, whilst at the *sn*-1 position about 61% of 18:1 was present in a total of 70.7% C-18 fatty acids located at this position. In addition there was about 21.6% of 16:1 fatty acid and 6.7% of 16:0 fatty acid esterified at *sn*-1 position. After 48h at 15^0, the C-16 chain length components in the SL fraction contributed 54% of the fatty acids, and at *sn*-2 16:0 represented 95.6% of the total. At the *sn*-1 position an increase of the sum of C-18 fatty acids was observed, largely associated with an increase in the relative proportion of 18:1.

5.6 Discussion

During the temperature adaptation response the stereospecific distribution of fatty acid at *sn*-1 and *sn*-2 of the glycerolipid found during growth at 28° was largely conserved with C-16 fatty acids predominant at *sn*-2 and C-18 fatty acids dominating at *sn*-1. The most prominent change brought about by the shift to lower temperature in the galactolipid fractions was an increase in 18:1 and 18:3 levels at *sn*-1, accompanied by an increase in 16:3 at both *sn*-1 and *sn*-2 of MGDG. The observed changes in the C-18 fatty acids at *sn*-1 are consistent with direct desaturation of C-18 acids occurring at *sn*-1 of pre-existing MGDG species to yield 18:3-MGDG. No significant increase in 18:3 in either the PG or SL fractions was apparent, but an increase in the proportion of 18:1 in the SL fraction was observed, suggesting that the accelerated synthesis of 18:1 and a replacement of 16:1 and 18:3 with the newly synthesized C-18 monoenoic fatty acid is an effect specific to this lipid class.

In contrast, in *A. variabilis*, during the first 10h following the downward temperature shift, there was a substantial decrease in the 16:0 in the MGDG fraction whilst there was a concomitant increase in 16:1 levels consistent with the direct desaturation of 16:0-MGDG to 16:1-MGDG (Sato and Murata, 1980); With further incubation at low temperature however, the relative contents of 16:0 and 16:1 fatty acids were almost restored to original level. In *Aphanizomenon* sp. 18:0 and 18:1 levels decreased and 18:3 increased in a similar way to *A.variabilis*, (Sato and Murata, 1980), consistent with direct desaturation of lipid–linked 18:0- and 18:1.These changes in the C-16 fatty acids at *sn*-2 and the C-18 fatty acids at *sn*-1 of the pre-existing MGDG species in *Aphanizomenon* to yield 18:3/16:1- and 18:3/16:3-MGDG would be expected to bring about an increase in the membrane fluidity allowing the organism to adapt to the lower temperature.

CHAPTER SIX

Studies of Lipid Metabolism in *Aphanizomenon* sp Using Radioactive Substrates

6.1 Introduction

Initial experiments described earlier in Chapter 3 established that *Aphanizomenon* sp. contains MGDG, DGDG, PG, and SL as major glycerolipid classes and the compositional studies described in Chapters 4 and 5 suggested possible biogenetic relationships. This chapter describes further studies on the pathway of lipid biosynthesis in *Aphanizomenon* sp. through use of radiolabelled glycerolipid precursors. Previous workers have used a range of radiolabelled substrates to investigate lipid metabolism in intact cells of cyanobacterial species (Nichols, 1968; Abreu-Grobois *et al.*, 1977 ; Sato and Murata 1982 a,b). Previous studies on *Anabaena variabilis* have indicated the pathway for the synthesis of glycerolipids and fatty acid desaturation by pulse-labeling experiments (Sato and Murata, 1981; 1982 a,b).

Initially the labelling pattern of the glycerolipids from [1-^{14}C]-acetate in *Aphanizomenon* sp. cells at 28° was established, and subseqently, in order to follow the changes in fatty acid desaturation, *Aphanizomenon* sp. cultures were labelled with [1-^{14}C]-acetate at 28° and the change in labelling pattern of glycerolipid classes, saturated and unsaturated fatty acids, and MGDG molecular species during temperature transition from 28° to 15° investigated. As described in Chapters 3 and 4, MGDG from *Aphanizomenon* sp. appeared to be the most significant lipid vehicle for temperature adaptation, and the labelling of the individual molecular species of MGDG was also studied in this chapter. In the isothermal studies at 28°, the radioactivity incorporated by cells from 10 day old cultures into the major lipid classes was determined following incubation for 1h, 3h, 6h, and 20h (Chapter 2.13.2). A parallel series of studies employing [1-(3)-

^{3}H]-glycerol was undertaken. This substrate was expected to label primarily the glycerol backbone of the glycerolipids, whilst ^{14}C-acetate was expected to be incorporated principally into the fatty acids of glycerolipids. Extraction and separation of the major glycerolipid classes and preparation of fatty acid methyl esters were carried out as previously described in Chapter 2. Radioactivity in lipid fractions and in samples recovered from TLC was determined by counting in the β-scintillation counter as described in Chapter 2.13.1. In the later part of this chapter the mechanism of the temperature-shift induced desaturation of the MGDG fraction was further investigated by a ^{3}H:^{14}C double labelling approach. *Aphanizomenon* sp cells were pre-labelled with ^{14}C-acetate and ^{3}H-glycerol at 28°, then subjected to a temperature shift to 15°, after which the ^{3}H:^{14}C ratios of the MGDG molecular species and fatty acids were determined to determine whether formation of highly unsaturated MDGD species occurs directly by desaturation of pre-existing saturated MGDG molecular species or involves *de novo* synthesis of MGDG species during the couse of the temperature adaptation response.

6.2 Incubation With Sodium [1-^{14}C] –Acetate at 28°

Cells suspensions were prepared as described in Chapter 2.13.2 and incubated with 40μCi of sodium [^{14}C]-acetate for the time indicated.

6.2.1 Labeling of the Total Lipid - and Fatty Acid Methyl Ester (FAME) fractions

Table 6.1 shows the relative percentage of the [1-^{14}C]-acetate incorporated into the total lipid and FAME fractions in *Aphanizomenon* sp. The amount of the ^{14}C-acetate substrate incorporated into the total lipid fraction was 0.86% after incubation for 1h, and reached 1.68% after 3h. Incorporation had not increased significantly after 6h, whilst after longer incubation, for 20h, the amount of ^{14}C-acetate appeared to decrease to 1.3%. The FAME fraction showed a similar pattern of incorporation. After 1h 0.78% of substrate radioactivity was incorporated into FAME, and the level increased to 1.08% and remained constant after 3h. After 6h there was no further increase, whilst the incorporation had decreased to 0.98% after 20h. The FAME fraction contained between 61-91% of the radioactivity incorporated into the total lipid fraction (Table 6.1). These data indicated that the total lipid and FAME fractions became substantially labelled within a 3h incubation period, and there was little significant increase in the labelling after longer incubation periods. This labelling patern in the total lipid and FAME fractions was observed in four replicate experiments.

6.2.2 Labelling of Individual Lipid Classes from Sodium [1-^{14}C] – Acetate

The incorporation of radioactivity into each individual major glycerolipid class separated by TLC in two separate incubations is shown in Tables 6.2.a and 6.2.b, Appendix 5, and Fig 6.1 shows the relative % distribution in each lipid over the labelling period studied for a representative experiment (Table 6.2a Appendix 5).

Table 6.1 Incorporation of sodium [^{14}C] – acetate into the total lipid and fatty acid methyl ester fractions.

Incubation Time (h)	Total Lipid *Radioactivity (DPM x10^6)	Total Lipid Radioactivity % of substrate	FAME *Radioactivity (DPM x 10^6)	FAME Radioactivity % of substrate	FAME as % Total lipid
1	0.75	0.86	0.69	0.78	91.0
3	1.48	1.68	0.94	1.08	63.7
6	1.53	1.74	0.95	1.08	60.9
20	1.15	1.30	0.86	0.98	74.6

*Average of duplicate aliquots.

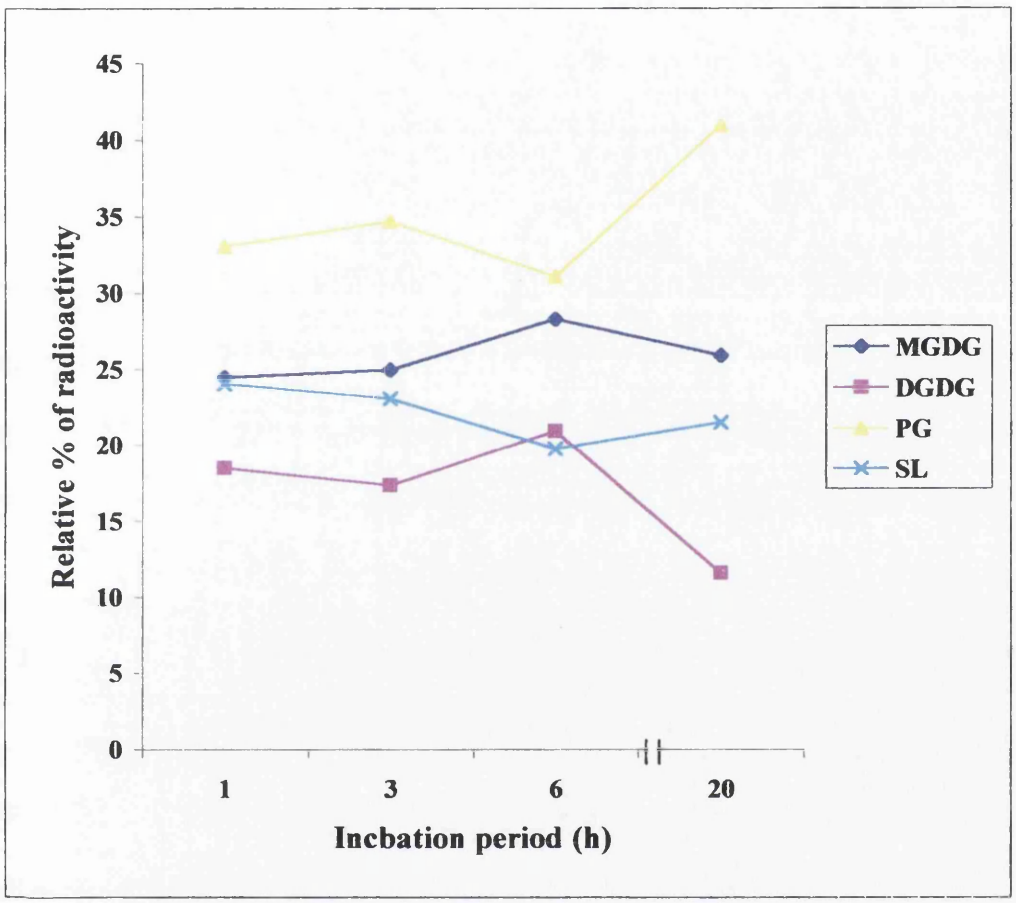


Fig 6.1 Relative percentage distribution of radioactivity in the major glycerolipid fractions in *Aphanizomenon* sp. labelled from sodium [^{14}C] -acetate.

After labelling for 1h at 28°, PG (33.0%) was the most heavily labelled lipid, whilst MGDG and SL contained approximately equal proportions of radioactivity (24.5% and 24.0% respectively), whilst DGDG contained the lowest relative proportion of radioactivity (18.5%). Between 1h and 3h the radioactivity remained relatively constant in all the lipid classes, but between 3h and 6h the label in the PG and SL fractions decreased (31.1% and 19.8% respectively) whilst radioactivity levels in MGDG and DGDG increased (28.3% and 20.9% respectively). In contrast to these relatively small changes in the labelling pattern during the first 6h of labelling, after 20h there was a substantial decrease in the proportion of label in DGDG and an increase in the radioactivity associated with PG (Fig 6.1).

6.3 Incubation with [1- ^{3}H]-Glycerol at 28°

The purpose of this experiment was to determine the incorporation of tritium labelled glycerol into the individual glycerolipid classes (MGDG, DGDG, PG, and SL). Cell suspensions were prepared as described in Chapter 2.13.2 and incubated with 200μCi of [1-^{3}H]-glycerol for the time indicated. The results reported are representative of three individual experiments.

6.3.1 Labelling of Total Lipid - and Fatty Acid Methyl Ester Fractions.

Table 6.4 shows the amount of ^{3}H-glycerol incorporated into the total lipid and FAME fraction of *Aphanizomenon* sp. during incubation at 28° for the indicated period. After 1h the total lipid contained 0.9% of the substrate radioactivity, and the incorporation of radioactivity did not increase significantly

Table 6.4 **Incorporation of radioactivity from [1-^{3}H]- glycerol into the total lipid and fatty acid methyl ester fraction of *Aphanizomenon* sp.**

Incubation Time (h)	Total lipid *Radioactivity (DPM x 10^6)	Total lipid Radioactivity % of substrate	FAME *Radioactivity after TLC (DPM x10^6)	FAME Radioactivity after TLC % of substrate	FAME Radioactivity % of total lipid
1	3.95	0.90	0.32	0.07	7.7
3	4.05	0.92	0.34	0.08	8.6
6	4.56	1.04	0.32	0.08	7.7
20	3.42	0.78	0.31	0.07	8.9

* Average of duplicate aliquots.

during the following 2h, but within 6h it had increased from 0.92% to 1.04%. During the following 14h incubation the incorporation of ^{3}H-glycerol decreased from 1.04 to 0.78%. The FAME fraction as initially isolated appeared to contained a relatively constant and high proportion of the substrate radioactivity at each time period (0.22-0.27%) of substrate radioactivity. Since ^{3}H-glycerol was expected to preferentially label the glycerolipid 'backbone' rather than the fatty acyl groups, further investigation of the radioactivity associated with the FAME fraction was carried out. The FAME fraction was subjected to radio-TLC using the solvent system described in Chapter 2.7.4b, and the FAME band visualised with primulin was recovered and the radioactivity determined by scintillation counting. The FAME band recovered in this way typically contained 29% of the original radioactivity applied to the plate and represented only 7% of the total lipid radioactivity. The results in Table 6.4 show that the 3h incubation period resulted in the substantial labelling of the total lipid fraction.

6.3.2 Labelling of Individual Lipid Classes

The distribution of radioactivity amongst the four major glycerolipid classes (Table 6.5, Appendix 5) is expressed in Fig 6.2 as the relative percent distribution within the individual lipid classes after labeling with ^{3}H-glycerol for 1h, 3h, 6h, and 20h. After incubation for 1h, PG was very substantially labelled (72.6% of total radioactivity), whilst similar levels (ca 10% of total) were found in each of MGDG, DGDG and SL. Between 1h and 3h the relative radioactivity in MGDG increased by 21.1%, whilst the radioactivity in PG decreased by 13.5%; there was no signifiant change in DGDG and SL radioactivity. Between 3h and 6h incubation with ^{3}H-glycerol, the relative radioactivity increased further in MGDG

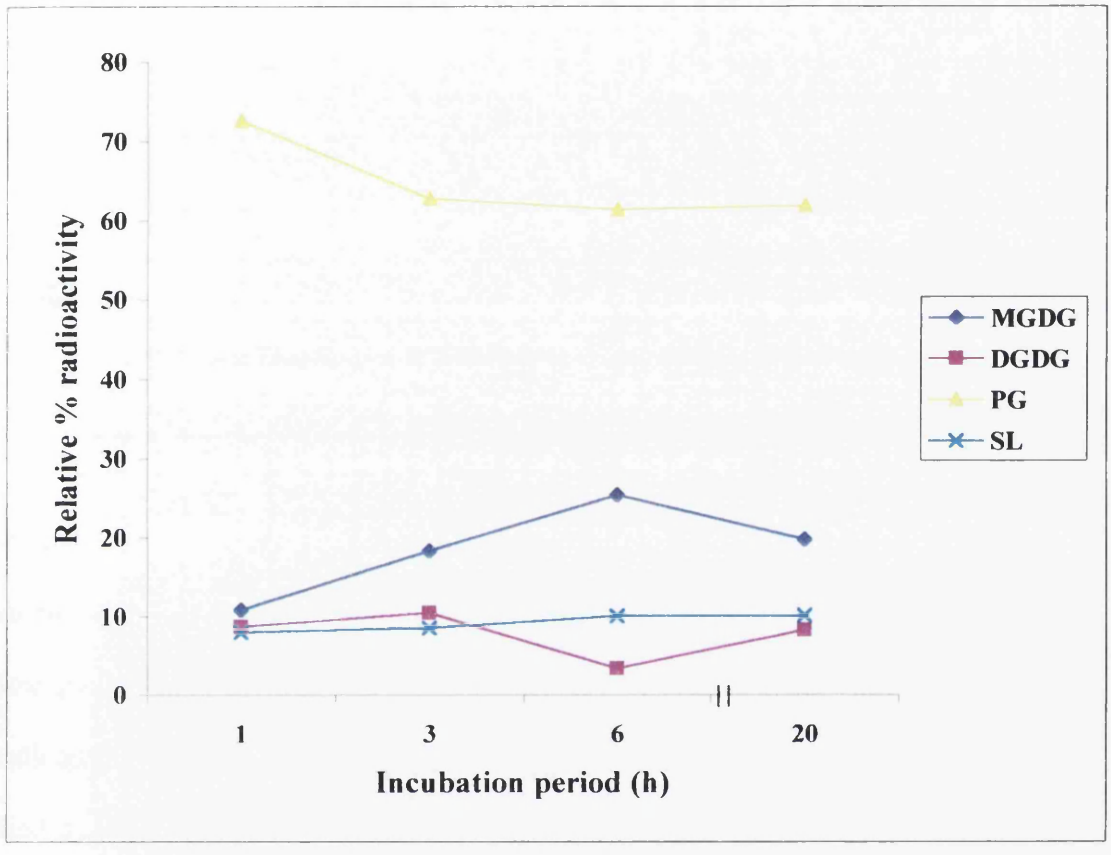


Fig 6.2 Relative percentage of distribution of radioactivity in the major glycerolipid fractions in *Aphanizomenon* sp. labelled from [1-^{3}H]- glycerol.

from 18.3% to 25.4%, whilst the proportion of radioactivity found in SL remained relatively constant. During this period there was a marked decrease (68.2%) in the proportion of radioactivity in DGDG. After a further 14h incubation period, MGDG had lost some 22% of its radioactivity whereas the proportion of label in DGDG recovered; the proportion of radioactivity in PG and SL remained essentially unchanged from that observed at 6h.

6.4 Discussion of Labelling Patterns in Total Lipid Classes and Fatty Acid Methyl Esters.

The incorporation of radioactivity from ^{14}C-acetate into the total lipid of *Aphanizomenon* sp after 3h was approximately double that seen after 1h, and remained fairly constant up to 6h, but decreased after longer incubation (20h). At all time periods examined from 1h onwards there was a substantial incorporation of radioactivity into the total lipid. The FAME fraction in *Aphanizomenon* sp. showed a similar pattern of incorporation and indicated that the *Aphanizomenon* lipids were heavily labelled within a 3h incubation period and that the extent of labelling was not increased by increasing the period of incubation. Whilst relatively little change was seen in the distribution of radioactivity in the glycerolipids after 1h or 3h incubation periods, small changes were observed after 6h and much more substantial changes occurred after 20h. After 6h incubation the level of radioactivity in both galactolipid fractions increased slightly, whilst the activity in SL decreased (Fig 6.1), but after 20h incubation the proportion of radioactivity in DGDG decreased sharply. These observations, together with the level of the respective total lipid incorporations (Table 6.1) indicated that a labelling period of 3h appeared to be an appropriate incubation period to obtain

near maximal labelling of glycerolipids and fatty acids. A feature of the the glycerolipid labelling pattern observed in *Aphanizomenon* sp was the relatively high level of radioactivity incorporated into PG at each incubation period. Abreu-Grobois *et al.*, (1977) in incubations of [1-^{14}C]-acetate for 3h with *A.cylindrica,* reported that PG contained only ca. 13% of total glycerolipid radioactivity whilst MGDG contained between 49%-66%, whilst in his earlier study in the same organism Nichols (1969) found that PG was the most highly labelled glycerolipid, but that the level of incorporation (25%-28%) was similar to that in the MGDG fraction (20-24%) following incubation with ^{14}C-acetate for periods between 0.75-20h. Using $NaH^{14}CO_3$ as a substrate, Sato and Murata (1982) found that in *A. variabilis* cultures after incubation for 0.1-1h the PG fraction contained 25-28% of the polar lipid radioactivity, whilst the monoglycosyldiglycerides contained some 60% of the total radioactivity. In the light of the relatively high proportion of radioactivity occurring in the PG fraction of *Aphanizomenon*, the analytical TLC of labelled- and un-labelled total lipid fractions was re-examined by use of an authentic PG standard, when the radioactivity in the fraction identified as PG clearly co-chromatographed with authentic PG visualized by the molybdenum blue phospholipid-specific detection reagent (see Chapter 2.7.2 b).

The incorporation of ^{3}H-glycerol radioactivity into the total lipid fraction (Table 6.4) followed a similar pattern to that observed for ^{14}C-acetate, with a 3h incorporation period providing near optimal labelling of the total lipid fraction. At all time periods examined PG was the most heavily labelled glycerolipid (Fig 6.2), but PG was most heavily labelled after the 1h incubation period, whilst the 3 glycoglycerolipids each contained approximately 10% of the total radioactivity. Between 1h-3h, whilst the radioactivity in SL and DGDG did not change

significantly, there was an increase in the labelling of MGDG which continued up to 6h, whilst between 3h-6h there was little change in SL labelling but a decrease in the level of label in DGDG. The small but significant levels of incorporation of radioactivity from ^{3}H-glycerol into the FAME fraction at each time period indicated that glycerol is significantly metabolized in *Aphanizomenon* sp and converted to fatty acid precursor such as acetyl CoA. The labelling pattern of the total lipid and the MGDG fraction from ^{3}H-glycerol indicated that an incubation period of 3h would be appropriate for subsequent double labelling studies.

6.5 Incorporation of Sodium [1-^{14}C]-Acetate into Monogalactosyldiacylglycerol Molecular Species

The culture conditions, labelling procedures, methods of total lipid extraction and analysis were the same as those described earlier in this chapter, except that a single incubation time of 3h was selected, as discussed above. The glycerolipid classes were separated by preparative silicagel-G TLC as described in Chapter 2.7.2a., and the relative distribution of radioactivity found in the lipid classes after 3h incubation is shown in Table 6.6. The MGDG fraction, containing 27.1% of the total glycerolipid radioactivity, was subjected to argentimetric-TLC and the molecular species of MGDG were separated by the method described in Chapter 2.7.4a. The separated molecular species were identified, and their fatty acid substitution pattern assigned by comparison of their Rfs with MGDG molecular species whose fatty acid composition had been characterised by gc as described in Chapter 2.7.4a . Table 6.7 shows the fatty acid substituents of the molecular species and the radioactivity among the MGDG fraction after

Table 6.6 Incorporation of radioactivity from sodium [^{14}C] - acetate into glycerolipid classes in *Aphanizomenon* sp. from the study of monogalactosyldiacyl-glycerol molecular species labelling pattern.

Lipid class	Radioactivity* dpm x 10^6	Relative radioactivity in each lipid class (%)
MGDG	0.37	27.10
DGDG	0.28	20.54
PG	0.42	30.93
SL	0.29	21.42

incubation with ^{14}C-acetate for 3h at 28°. The 18:1/16:1 molecular species was the most heavily labelled fraction (59.1% of MGDG radioactivity), whilst the 18:1/16:0 monoenoic species was also substantially labelled (36.6% radioactivity). Relatively low amounts of radioactivity were detected in the species of MGDG containing polyunsaturated C-18 fatty acid. 18:3/16:0 (2.1%), 18:3/16:1 (0.4%), and 18:2/16:0 (1.7%).

6.6 Incorporation of Sodium [1-^{14}C] Acetate into Saturated and Unsaturated Fatty Acid Classes

The FAME fraction from the total lipid extract from *Aphanizomenon* cells labelled for 3h with ^{14}C-acetate at 28° was separated on the basis of the number of double bonds present by argentimetric-TLC, as described in Chapter 2.7.4b. Table 6.8 shows the distribution of radioactivity in the fatty acids. Most of the radioactivity was found in the band containing the saturated fatty acids (16:0, 18:0), which represented 49.9% of the total radioactivity. The monoenoic fatty acid band, comprised 16:1 and 18:1, contained 40.1% of the radioactivity, whilst relatively low levels of radioactivity were found in the bands containing dienoic (16:2, 18:2) and trienoic (16:3, 18:3) fatty acids, with 3.0% and 7.0% of total radioactivity respectively. These results indicated that saturated fatty acids and the fatty acids containing one double bond were the primary fatty acids synthesised in cultures grown at 28°, and are consistent with the preponderance of label in MGDG species containing only saturated- or monoenoic fatty acids (Table 6.7).

Table 6.7 Distribution of radioactivity in molecular species of monogalactosyldiacylglycerol labelled from sodium [1-^{14}C] - acetate

*Band no.	Rf on $AgNO_3$ TLC	Predominant fatty acid composition	Relative % distribution of radioactivity
4	0.58	18:3 &16:1	0.35
5	0.63	18:3 &16:0	2.12
6	0.70	18:2 & 16:0	1.72
7	0.75	18:1 &16:1	59.05
8	0.80	18:1 & 16:0	36.76

***Bands on $AgNO_3$ plate are designated from 4 (lowest Rf) to 8 (highest)**

Table 6.8 Incorporation of radioactivity from sodium [1-^{14}C] - acetate into saturated and unsaturated fatty acid classes in *Aphanizomenon sp.*

*Band no.	Rf on AgNO3-TLC	Fatty acid class	Total radioactivity dpm x 10^6	% of total fatty acid radioactivity
1	0.88	Saturated	0.41	49.86
2	0.54	Monoenoic	0.33	40.11
3	0.28	Dienoic	0.02	3.00
4	0.09	Trienoic	0.06	7.04

*** Bands on $AgNO_3$-TLC plate are designated from 1 (highest Rf value) to 4 (lowest Rf value).**

6.7 Effect of Reducing Culture Temperature to 15° on Labelling Pattern of Lipids in *Aphanizomenon* Cells Prelabelled with Sodium [1-^{14}C]-Acetate at 28°.

Cultures of *Aphanizomenon sp.* were grown for 10 days at 28°, then the cells were harvested and pre-labelled for 3h at 28° with ^{14}C-acetate. The excess of ^{14}C-acetate substrate was removed by washing the resuspended cells, and 10mM unlabelled sodium acetate was added to dilute out any remaining radiolabelled substrate (see Chapter 2.13.3); the cells were then incubated at 15° for the appropriate time period.

6.7.1 Effect of 28° to 15° Temperature Transition on the Glycerolipid Labelling Pattern

The radioactivity found in each lipid class in the 48h following the temperature transition from 28° to 15° is shown in Table 6.9. The amount of the radioactivity in the total glycerolipid fraction decreased by 32.3% during the first 12h following the temperature transition and there was no further decrease at the 24h sampling period, whilst an increase was apparent after 48h (Table 6.9). These observations are consistent with the explanation that *de novo* biosynthesis of glycerolipid from radiolabelled precursors did not occur for upto 24h after the substrate acetic acid was removed, and unlabelled acetic acid added to the incubation system. Following the temperature shift to 15° (12h, 24h, and 48h), there was a significant difference in the relative distribution of radioactivity between 0h and 12h, but after 24h there was a significant increase

Table 6.9 The distribution of radioactivity in lipid classes in cells of *Aphanizomenon* sp labelled with sodium [1-^{14}C] - acetate following a temperature shift from 28° to 15°.

Lipid class	Radioactivity (dpmx10^6) Time at 15° (h) 0†	12	24	48
MGDG	0.13	0.08	0.13	0.09
DGDG	0.03	0.02	0.04	0.06
PG	0.12	0.08	0.04	0.05
SL	0.06	0.06	0.05	0.04
Total glycerolipid	0.34	0.24	0.26	0.24

†Cells preincubated with sodium [1-^{14}C]- acetate at 28° for 3h.

in MGDG (23.6%) and decrease in PG (18.3%) and SL (14.8%), whilst DGDG was not significantly changed relative to zero time (Table 6.9). After 48h the pattern had changed, with a fall in MGDG radioactivity between 24h and 48h of 31.6% and increases in DGDG (24.7%) and PG (46.5%) whilst SL radioactivity remained constant over the same time period.

6.7.2 Distribution of Radioactivity from [1-^{14}C]-acetic Acid in Monogalactosyldiacylglycerol Molecular Species Following 28° to 15° Temperature Transition

The MGDG molecular species of MGDG were separated by the method described in (Chapter 2.7.4a), and the distribution of radioactivity in MGDG molecular species after temperature shift from 28° to 15° is shown in Table 6.10. The fatty acid stereospecific distribution (*sn*1/*sn*2) within the MGDG molecular species was assigned on the basis of the stereospecific analysis of the unlabelled MGDG fraction described in Chapter 5. As observed earlier (Table 6.6), after incubation for 3h at 28° the most heavily labelled MGDG molecular species was 18:1/16:1-MGDG. After 12h from the temperature down-shift, the radioactivity in 18:1/16:1 decreased substantially from (67.7 to 29.7%), whilst the label in 18:2/16:0-MGDG increased from 13.2% to 36.9% and in 18:3/16:0-MGDG from 7.8% to 24.5%. During the next 12h a further sharp decrease in the radioactivity was seen in 18:1/16:1-MGDG accompanied by a smaller decrease in 18:2/16:0-MGDG, whilst during the same period increases in 18:3/16:0-MGDG and 18:3/16:1-MGDG were detected. After 48h incubation at 15° most of the radioactivity was found in 18:3/16:0, and it can be observed that the radioactivity in 18:3/16:0 increased from 7.8% at 28° to 46.0% after 48h at

Table 6.10 The distribution of sodium [1-^{14}C]-acetate radioactivity in MGDG following 28° to 15° temperature transition

*Band no.	Rf on $AgNO_3$-TLC	Distribution of Fatty acids in MGDG species	Relative % of radioactivety Time at 15° (h) †0	12	24	48
4	0.58	18:3 / 16:1	1.1	4.6	10.7	17.7
5	0.63	18:3 / 16:0	7.8	24.5	34.4	46.0
6	0.70	18:2 / 16:0	13.2	36.9	28.9	27.2
7	0.75	18:1 / 16:1	69.7	29.7	17.8	9.1
8	0.80	18:1 / 16:0	7.3	4.3	8.3	nd

***Bands on $AgNO_3$ - TLC plate are designed from 4 (lowest Rf) to 8 (highest Rf).**

† 0 Cells preincubated with sodium [1-^{14}C]- acetate at 28 for 3h.

15°. On the other hand the radioactivity in 18:1/16:1 was greatest at 28°, but was the least heavily labelled of the MGDG molecular species detected after 48h at 15°.

6.7.3 Distribution of Radioactivity from Sodium[1-^{14}C]-Acetate in Saturated and Unsaturated Fatty Acid Classes

The FAME fraction obtained from the total lipid extract was separated by the method described earlier in Chapter 2.7.4b. The distribution of radioactivity in saturated and unsaturated fatty acid classes following the temperature shift from 28° to 15° is shown in Table 6.11. The distribution of radioactivity within the fatty acid classes in the 0h sample, following pre-incubation at 28°, was in good agreement with that observed in the preliminary experiment described earlier (Table 6.7). The saturated fatty acids were the most heavily labelled fraction (46.4% of fatty acid radioactivity), whilst amongst the unsaturated fatty acids, the monoenoic fatty acids were heavily labelled (45.4% of the radioactivity), whilst the polyunsaturated fatty acids contained much smaller amounts of radioactivity; the dienoic fatty acids had the lowest radioactivity (1.2%) with the trienoic fatty acid fraction containing 8.0% of total radioactivity. Within 12h at 15° the relative proportion of radioactivity in the saturated fatty acid fraction had decreased by 16%, and there were concomitant increases in the dienoic and trienoic fatty acid fractions. A further substantial increase in the trienoic fraction was seen after 24h, accompanied by decrease in the saturated and dienoic fractions. There was relatively little change apparent of radioactivity in the monoenoic fatty acid fraction over the period examined. This labelling pattern was maintained after 48h at 15°.

Table 6.11 The distribution of radioactivity in saturated and unsaturated fatty acid fractions from the total lipid of cells of *Aphanizomenon* sp. labelled with sodium [1-^{14}C] - acetate following a temperature shift from 28° to 15°

			% of fatty acid radioactivity			
*Band no.	Fatty acid fraction	Rf		Time at 15° (h)		
			0†	12	24	48
1	Saturated	0.88	46.4	39.0	30.8	25.3
2	Monenoic	0.54	45.4	45.6	42.9	39.8
3	Dienoic	0.28	1.2	6.1	1.6	1.7
4	Trienoic	0.09	8.0	9.3	20.7	33.3

* Bands on $AgNO_3$-TLC plate are designated from 1 (highest Rf value) to 4 (lowest Rf value).

†Cells preincubated with sodium [1-^{14}C] - acetate at 28° for 3h

6.8 Discussion of the Labelling Patterns for Monogalactosyldiacyl glycerol Molecular Species and Saturated and Unsaturated Fatty Acid Classes

At 28° the monoenoic-(18:1/16:0) and (18:1/16:1) dienoic molecular species of MGDG were the most heavily labelled (Table 6.7), in a pattern which was in agreement with the distribution of fatty acid radioactivity predominantly in the saturated and monoenoic fatty acid fractions (Table 6.8). The pattern of radiolabelling of the glycerolipid classes following a temperature transition from 28° to 15° indicated that under the conditions used there was no net synthesis of new glycerolipid for at least the first 24h of the temperature adaptation process (Table 6.9). During the first 12h of the transition period the level of radioactivity in the MGDG fraction initially declined, but then remained approximately constant between 12h-24h. Analysis of the MGDG molecular species during the temperature adaptation process indicated that the proportion of label in the 18:1/16:1 MGDG species showed a dramatic decrease, with concomitant increase in the trienoic and tetraenoic MGDG species (Table 6.10). In the absence of new synthesis of glycerolipid, these data are consistent with direct desaturation of this dienoic MGDG species as the major source of the more highly unsaturated MGDG molecular species 18:3/16:1. However direct desaturation of 18:1/16:0 could not fully account for formation of 18:2/16:0 and 18:3/16:0 seen under these conditions, suggesting the operation of an alternative route for formation of species containing polyunsaturated fatty acids. Analysis of the FAME fraction from the total lipid fraction provided further evidence for a marked increase in the proportion of 18:3 between 12h-48h following temperature transition. This analysis also revealed a concomitant decrease in the monoenoic fatty acid

fraction, but this effect was not as large as that observed in the fatty acids of the MGDG fraction. In the total lipid, the saturated fatty acid fraction showed the largest decrease concomitant with the increase in 18:3. The studies of MGDG labelling during temperature transition described above suggest that direct desaturation of 18:1 in MGDG species to 18:3 may make a significant contribution during temperature adaptation, but also indicated that an alternative additional pathway may operate in formation of MGDG species containing polyunsaturated fatty acids.

Studies on the changes in labelling pattern within the total lipid fatty acid fraction indicated that a concomitant decrease in saturated fatty acid, rather than monoenoic fatty acid, was associated with an increase in trienoic fatty acid. These observations suggest that changes in glycerolipid species other than MGDG, in which the major effect was seen in the monoenoic fatty acid fraction, make a substantial contribution to temperature adaptation in *Aphanizomenon* sp.

6.9 Investigation of the pathway of MGDG Formation by Dual Labelling with Sodium [1-^{14}C]-Acetate and [1-(3)-^{3}H]-glycerol

The aim of this experiment was to attempt to evaluate, using a different experimental approach to the use of stable isotopes and mass spectrometry which had been used to study this problem in *A. variabilis* (Murata *et al.*, 1981; 1982 a; b), whether desaturation of pre-existing MGDG species represented the principle route of formation of highly unsaturated MGDG species in *Aphanizomenon* during a high to low temperature transition.

6.10 Incubation with Sodium [1-^{14}C]–Acetate and [1-(3)-^{3}H-Glycerol] Following Temperature Shift from 28° to 15°.

Aphanizomenon cell suspensions were prepared as described in Chapter 2.13.4 and incubated with 40 μCi of sodium[^{14}C] – acetate and 200 μCi of [^{3}H] – glycerol for the time indicated. The excess of ^{14}C-acetate substrate was removed by washing the resuspended cells, and 10mM unlabelled sodium acetate was added to dilute out any remaining radiolabelled acetic acid substrate (see Chapter 2.13.3).The dual labelled lipid samples were analysed according to Scheme 2.7.

6.10.1 Labelling of the Total Lipid and Fatty Acid Methyl Ester (FAME) Fractions

Table 6.12 shows ^{3}H-radioactivity (dpm) and ^{14}C-radioactivity (dpm) together with the ^{3}H:^{14}C dpm ratios and the quench values (H$^{\#}$) in the the total lipid and FAME fractions in *Aphanizomenon* sp. The amount of the ^{14}C-acetate substrate incorporated into the total lipid fraction was increased by about 50% after the first 12h following the downward temperature shift. Between 12h and 24h at 15°, the radioactivity (dpm) remained relatively constant, whilst the incorporation of ^{3}H-glycerol also increased significantly in the first 12h following the temperature shift, and declined slightly over the following 12h. The increase in the total lipid label after the first 12h incubation, indicated that *de novo* lipid biosynthesis from ^{14}C-acetate and ^{3}H-glycerol occurred during this period, possibly from a pool of intermediates, e.g. acetyl-CoA and phosphatidic acid that had built up during the pre-labelling period. Table 6.12 also shows the ^{14}C-acetate radioactivity (dpm) found in the fatty acid fraction during the same period.

Table 6.12 Incorporation of radioactivity from ^{3}H-glycerol and ^{14}C – acetate into the total lipid and fatty acid methyl ester in *Aphanizomenon* sp after temperature shift from 28° to 15°.

Total Lipid

Temperature shift Period (h)	^{3}H- radioactivity (dpm)	^{14}C – radioactivity (dpm)	^{3}H : ^{14}C (dpm) ratio	H#
0	64306	5890	10.9: 1	146.9
12	181055	8786	20.6:1	190.3
24	145016	7318	19.8:1	184.2

FAME

Temperature shift Period (h)	^{3}H- radioactivity (dpm)	^{14}C – radioactivity (dpm)	^{3}H : ^{14}C (dpm) ratio	H#
0	5005	1636	3.1:1	107.6
12	3127	2547	1.2:1	109.4
24	4808	2570	1.9:1	106.4

The FAME ^{14}C-radioactivity also suggests that there may have been continued *de novo* biosynthesis of fatty acids during the first 12h, but not during 12-24h, broadly in agreement with the result for total lipid. The difference in the ^{3}H:^{14}C dpm ratios in total lipid fraction between 0h and 12h suggested that *de novo* lipid biosynthesis occurred during the first 12h following the temperature shift from 28° to 15°, whilst the similarity of the ratios of the samples at 12h and 24h, was consistent with the view that *de novo* synthesis had largely ceased.

6.10.2 Labeling of Individual Glycerolipid Classes from [1-^{14}C] – Acetic Acid and [1-^{3}H]–Glycerol Following the Temperature Shift from 28° to 15°.

The radioactivity (dpm) in each of the individual glycerolipid classes, isolated by TLC, during the period following the temperature shift from 28° to 15° is shown in Tab.6.13 and the dpm ratio of each glycerolipid is shown in Fig. 6.3. Twelve hours after the temperature transition the ^{3}H:^{14}C dpm ratio of MGDG increased from 2.0:1 to 2.5:1 whilst during the following 12h period there was no increase in the ratio (Fig 6.3). The MGDG radioactivity (Table 6.13) increased in the first 12h of the transition but remained relatively constant in the following incubation period. The ^{3}H:^{14}C dpm ratio of DGDG also increased during the first 12h incubation period (Fig. 6.3) from 3.3:1 at 0h to 4.5:1 after 12h whilst no significant increase could be detected after that period. The incorporation of ^{14}C radioactivity into DGDG increased in the first 12h, whilst between 12h and 24h the incorporation declined slightly (Table 6.12). At 0h the ratio of ^{3}H:^{14}C dpm in the PG fraction at 33.5:1 was the highest of all the lipid classes (Fig. 6.3).

Table 6.13 Incorporation of radioactivity from ^{3}H-glycerol and ^{14}C – acetate into the glycerolipid classes of *Aphanizomenon* sp after temperature shift from 28° to 15°.

MGDG

Temperature shift Period (h)	^{3}H- radioactivity (dpm)	^{14}C – radioactivity (dpm)	^{3}H : ^{14}C (dpm) ratio	H#
0	5309	2669	2.0:1	126
12	9047	3609	2.5:1	127
24	6494	3449	1.9:1	124

DGDG

Temperature shift Period (h)	^{3}H- radioactivity (dpm)	^{14}C – radioactivity (dpm)	^{3}H : ^{14}C (dpm) ratio	H#
0	1937	580	3.3:1	112
12	3612	810	4.5:1	114
24	3230	782	4.1:1	110

PG

Temperature shift Period (h)	^{3}H- radioactivity (dpm)	^{14}C – radioactivity (dpm)	^{3}H : ^{14}C (dpm) ratio	H#
0	4297	1283	33.5:1	107
12	48423	1888	25.7:1	109
24	23068	1641	14.1:1	108

SL

Temperature shift Period (h)	^{3}H- radioactivity (dpm)	^{14}C – radioactivity (dpm)	^{3}H : ^{14}C (dpm) ratio	H#
0	5573	2858	2.0:1	108
12	13315	5238	2.5:1	111
24	4882	2667	1.8:1	108

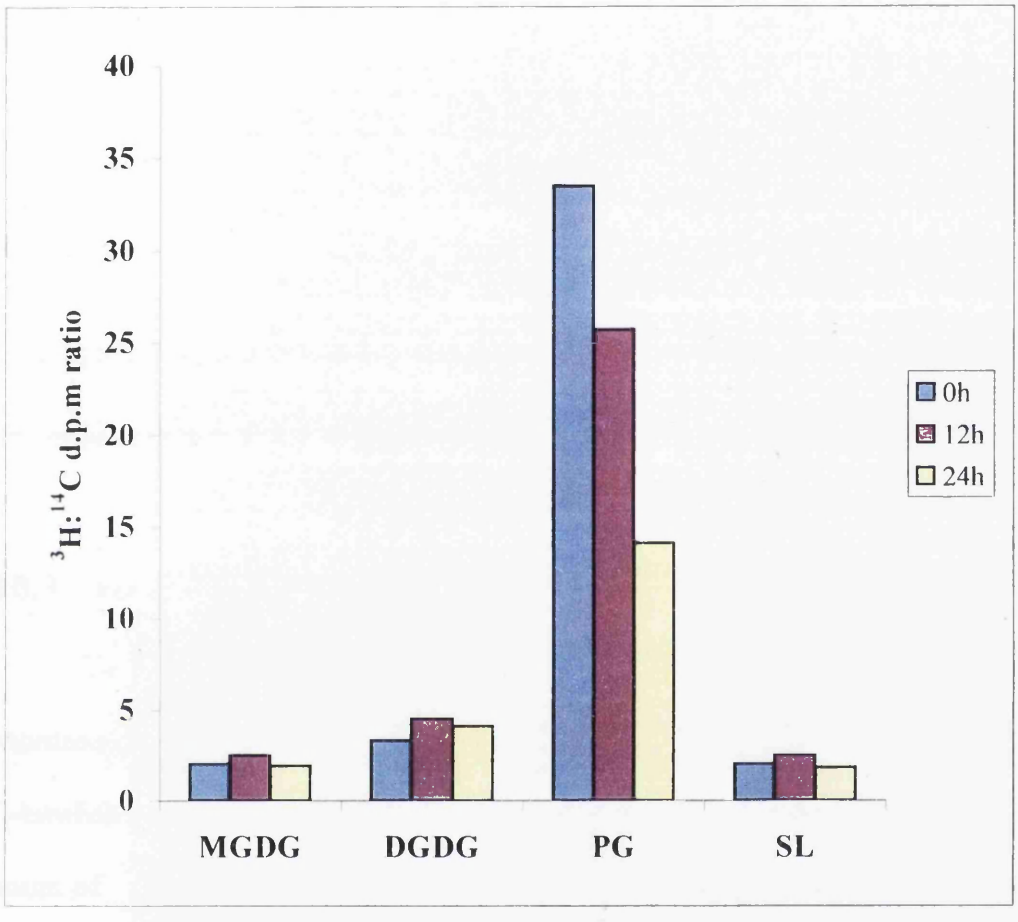


Figure 6.3 $^3H:^{14}C$ dpm ratios of *Aphanizomenon* sp. glycerolipids during a 28° to 15° temperature shift.

After 12h incubation at 15°, the ratio of ^{3}H:^{14}C decreased to reach 25.7:1, and during the following 12h period the ratio continued to decline to 14.1:1. The amount of ^{14}C-radioactivity in the PG fraction increased significantly by (47%), during the first 12h whilst between 12h and 24h the incorporation decreased (Table 6.12). In the SL fraction from *Aphanizomenon* sp. after incubation for 12h at 15° the ratio of ^{3}H:^{14}C radioactivity increased from 2.0:1 to 2.5:1, but after a further 12h incubation period, the ratio decreased to 1.8:1 (Fig 6.3). The amount of ^{14}C- radioactivity in the SL fraction increased in the first 12h of the transition period, but it decreased between 12h and 24h to a level below that seen at the start of the experiment (Table 6.12).

6.10.3 ^{3}H:^{14}C Radioactivity Ratios of MGDG Molecular Species

Table 6.14 shows the ^{3}H:^{14}C dpm ratios of MGDG molecular species in *Aphanizomenon* sp. together with the radioactivity incorporated from both ^{3}H and ^{14}C-labelled substrates. After 12h incubation at 15°, there was an increase in the amount of ^{14}C radioactivity in both the 18:3/16:0 and 18:3/16:1 MGDG molecular species, but the amount of activity in 18:1/16:0 and 18:1/16:1 either remained constant (18:1/16:0) or had increased (18:1/16:1) from the level present at 0h. The formation of the more unsaturated molecular species during this period could not therefore be accounted for exclusively by the direct desaturation of monoenoic- and dienoic molecular species, and some new synthesis must have occurred in this 12h period, as previously indicated by the pattern of the total lipid and total MGDG fraction (Tabs. 6.12 and 6.13). After the second 12h of the temperature transition period, the radioactivity in the polyunsaturated MGDG species had increased relative to the level after the first 12h, and there was a

Table 6.14 Incorporation of radioactivity from ^{3}H-glycerol and ^{14}C-acetate into the MGDG molecular species in *Aphanizomenon* sp after a temperature shift from 28° to 15°.

0h at 28°

Molecular species	^{3}H : ^{14}C dpm ratio	^{3}H- radioactivity dpm	^{14}C – radioactivity dpm	H#
18:3/16:1	5.5:1	169	31	122
18:3/16:0	4.0:1	483	122	124
18:2/16:0	3.8:1	2227	583	120
18:1/16:1	4.5:1	3159	706	124
18:1/16:0	3.8:1	1145	306	121

12h at 15°

Molecular species	^{3}H : ^{14}C dpm ratio	^{3}H- radioactivity dpm	^{14}C – radioactivity dpm	H#
18:3/16:1	4.8:1	358	75	120
18:3/16:0	4.3:1	1813	423	124
18:2/16:0	3.9:1	3692	940	121
18:1/16:1	2.9	3586	1254	118
18:1/16:0	4.1:1	1310	321	125

24h at 15°

Molecular species	^{3}H : ^{14}C (dpm) ratio	^{3}H- radioactivity (dpm)	^{14}C – radioactivity (dpm)	H#
18:3/16:1	4.3:1	940	221	122
18:3/16:0	5.3:1	3270	620	119
18:2/16:0	4.4:1	5794	1304	118
18:1/16:1	4.8:1	2916	608	116
18:1/16:0	6.1:1	468	76	119

concomitant decrease in the level of radioactivity in the 18:1/16:1- and 18:1/16:0-MGDG species (Table 6.14). The ^{3}H:^{14}C dpm ratios of the 18:3/16:1- and 18:2/16:0-MGDG species at the end of the second 12h period were similar to that of the 18:1/16:0-MGDG species after 12h of the transition, suggesting that these polyunsaturated MGDG species may have arisen by direct desaturation of pre-existing 18:1/16:0-MGDG.

6.11 Discussion of the Dual Radio-labelling Investigation of MGDG Metabolism During a Temperature Transition from 28° to 15°

The incorporation of radioactivity from both ^{3}H-glycerol and ^{14}C-acetate in the total lipid fraction of *Aphanizomenon* sp. increased in the first 12h following the downward temperature shift, whilst the radioactivity remained relatively constant in the following incubation period (Table 6.12). The increase in the radioactivity during the first 12h incubation period indicated that under the experimental conditions *de novo* lipid biosynthesis from ^{14}C-acetate and ^{3}H-glycerol continued during the first 12h following downward temperature shift. The continued synthesis of glycerolipids during the first 12h of the transition in the dual labelling study was in contrast to the results from the study using ^{14}C-acetate alone described above, in which there was no significant increase in labelling levels during the first 12h of the transition (Table 6.9). Washing of the *Aphanizomenon* cells and addition of 10mM unlabelled acetate under these conditions appeared to be sufficient to prevent *de novo* synthesis from the radiolablled precursor during temperature transition. It appears that in the dual labelling experiment, washing of the cells and addition of 10mM sodium acetate

was insufficient to prevent biosynthesis of new glycerolipid species from the labelled precursors during the first 12h of the transition. It may be that a substantial pool of phosphatidic acid formed during the pre-labelling period in the presence of ^{3}H-glycerol did not become depleted until some 12h into the temperature transition. The glycerolipid labelling pattern observed in *Aphanizomenon* sp. (Table 6.13) shows that the level of radioactivity and the ^{3}H:^{14}C dpm ratios of the MGDG, DGDG and SL fractions increased during the first 12h of the temperature down shift, whilst after that period the level of radioactivity fell slightly, as did their ^{3}H:^{14}C dpm ratios. At all times, the ^{3}H:^{14}C ratio of DGDG was significantly higher than those of MGDG and SL. This may reflect incorporation of ^{3}H-glycerol into the galactose moiety transferred to an acceptor MGDG during DGDG biosynthesis (Chapter 2.13.3). The ^{3}H:^{14}C dpm ratio of the PG fraction was very substantially higher than that of the glycolipid fractions at all time periods examined (Table 6.13). Based on the presence of two glycerol moieties in its structure, a higher ^{3}H:^{14}C dpm ratio for PG than for the glycolipids was predicted, but the values obtained, which were ×10 – ×15 higher than for the glycerolipid, were unexpectedly high. This may suggest that during metabolism of [1-^{3}H]–glycerol to phosphatidic acid loss of tritium label occurs, whilst the glycerol moiety which provides the PG head group is not subject to ^{3}H-exchange.

The compositional and ^{14}C-acetate radiolabelling studies in *Aphanizomenon* sp described above provided evidence that during adaptation to lower temperature highly unsaturated MGDG molecular species may be formed directly from more saturated MGDG molecular species. Direct desaturation of lipid-bound fatty acyl groups during temperature adaptation has been established in *A.variabilis* (Sato *et*

al., 1986). In the latter study cyanobacterial cells were pre-labelled with [^{13}C]Na_2CO_3, and mass spectrometric analysis of the distribution of ^{13}C in the 2-acylglycerol moieties derived from MGDG indicated that palmitic acid (16:0) was converted to palmitoleic acid (16:1(9)) *in vivo* by lipid linked desaturation, but not *via* a pathway consisting of deacylation, desaturation and reacylation. To further investigate the mechanism by which highly unsaturated glycerolipids are formed in *Aphanizomenon* sp. a dual radioisotope labelling approach was used, and the ^{3}H:^{14}C dpm ratios of the major MGDG molecular species determined. This approach had not previosly been employed to study this phenomenon and in principle requires much less sophisticated analytical procedures that the mass spectrometric approach. It was found that, despite the procedures employed to limit *de novo* glycerolipid biosynthesis during the temperature transition, the incorporation of radioactivity from ^{3}H-glycerol and ^{14}C-acetate as well as the ^{3}H:^{14}C dpm ratio both increased during the first 12h of adaptation in all MGDG molecular species, indicating that *de novo* glycerolipid formation continued during this period. However between 12h-24h of the transition period, the observed increase in the radioactivity in MGDG species containing PUFAs and commensurate decrease in the radioactivity in the MGDG species containing only saturated or monoenoic fatty acids, together with the similarity in ^{3}H:^{14}C dpm ratio of the polyunsaturated MGDG species after 24h with that of the 18:1/16:0-MGDG species after 12h, provided support for the view that lipid-linked desaturation of C-16 and C-18 fatty acids occurs during temperature adaptation in *Aphanizomenon* sp.

CHAPTER SEVEN

General Discussion

The study of the growth characteristics and fatty acid composition of *Aphanizomenon* sp. established that under the culture conditions the organism reached mid-late exponential phase after 10-12 days. The fatty acid composition at both 28° and 15° growth temperature was consistent with the classification of *Aphanizomenon* sp. as belonging to Group 2 under the classification system of cyanobacteria by their fatty acid composition (Kenyon 1972; Kenyon *et al.*, 1972). However the fatty acid composition of *Aphanizomenon* sp. was distinguished by the presence of a small but significant amounts (ca. 3%) of 16:3 in the organism when cultured at 28°, and a substantial (ca. 13%) level in the cultures grown at 15°. This fatty acid has not been detected in a range of other Group 2 cyanobacteria when grown either at 'high' or 'low' temperature (Murata *et al.*, 1992). Comparison of the fatty acid composition from cells grown isothermally at 28° with that from cells grown at 15° indicated that at higher temperature the saturated fatty acid 16:0 was the major fatty acid and occurred at a concentration approximately double that of 18:3, whereas at 15° 18:3 was the major fatty acid, and was present at approximately double the level of 16:0 (Tables 3.1 and 3.2). Growth at 15° also led to the appearance of substantial (ca. 13%) levels of an additional trienoic fatty acid, 16:3, which together with increased proportion of 18:3 would be expected to significantly increase the fluidity of the membranes in cultures grown at 15° relative to those grown at 28°. These temperature – dependent fatty acid profiles presumably reflect adaptations which allow the organism to maintain physiological function and to grow at the extremes of this temperature range, which would be experienced by colonies of the natural population of *Aphanizomenon* in the Baltic Sea during the year.

In response to a downward temperature transition of similar extent to that which would be experienced by *Aphanizomenon* cells descending from the surface to

the thermocline, following, for example, a wind-mixing event, the relative proportion of 18:3 and 16:3 increased in the galactolipids and SL whilst the level of the less unsaturated fatty acids decreased (Fig. 4.4). In the PG fraction however, a quite different response was seen in which there was a substantial increase in the relative proportion of 18:1, whilst saturated and polyunsaturated fatty acid levels fell (Fig. 4.4). The time course of the response indicated that in MGDG specifically there was a decrease in 16:0 and 18:1 and increase in 18:3 which started to occur within 12h of the temperature transition, after which the increase in 18:3 was sustained, but at a lower rate (Fig. 4.2). Notably in the 12h-24h period, coincident with the continuing decrease in 16:0, the level of 16:3 increased substantially and threafter remained at a constant level. If thus appears that in this lipid class the major response is a sustained increase in 18:3 initiated within 12h of the temperature transition, superimposed on which is an increase in 16:3, specifically between 12h and 24h of the temperature transition. The adaptive response of *Aphanizomenon* sp. is thus in contrast to that observed in *A. variabilis* in which a rapid transient increase in 16:1 levels was observed within 12h of temperature reduction in the MGDG fraction, followed by a second slower sustained phase in which 18:3 accumulated whilst 18:1 and 18:2 levels fell (Sato and Murata, 1980). The pattern seen in the MGDG fraction could be seen in the total lipid fraction, suggesting that the MGDG fraction has a major role in temperature adaptation in *Aphanizomenon.* Following upward temperature transition from 15° to 28°, the most significant effects were a substantial increase in 16:1 and a smaller increase in 18:1, accompanied by a decrease in 16:3 and 18:3. These changes however do not occur for the first 12h following temperature transition. The time scale of the changes in fatty acid composition observed on temperature transition from 15° to 28° is consistent with a physiological

significance in temperature adaptation during upward migration of *Aphanizomenon* colonies in the Baltic. In a study of the vertical population of the *Aphanizomenon* flos-aquae population on a drift station east of Bornholm Island (Walsby *et al.*, 1997), wind induced mixing of *Aphanizomenon* colonies resulted in dispersion of the cyanobacterial population down to 18m, just above the summer thermocline at 21m. During the following four relatively calm days, the colonies floated up at a rate of approximately $1.5m.d^{-1}$, consistent with the view that the cyanobacterial cells would have sufficient time to adapt their fatty acid composition whilst returning to the surface layer. These processes would also be an important adaptive mechanism as the average sea temperature increased during the summer. During this upward temperature transition the changes in the PG fraction were broadly consistent with those seen in the galactolipid and SL fractions.

Irrespective of growth temperature, in both the galactolipid fractions and the SL fraction, C-18 fatty acids were essentially absent from the *sn*-2 position, whilst dominating at the *sn*-1 position, in a pattern consistent with that reported in other Group 2 cyanobacteria (Murata *et al.*, 1992). The C-16 fatty acids were almost entirely located at *sn*-2 in all lipid classes. The stereospecific distribution of fatty acids in PG was however markedly different from that in the other lipid fractions, in that a substantial amount of 18:0 was present at *sn*-2 at 28°, whilst C-16 fatty acids, in particular 16:1 were substantial components at *sn*-1 in PG from cultures grown at 15°. This point of difference further distinguished the characteristics of the PG fraction from the other glycerolipids in *Aphanizomenon*. During adaptation following a downward temperature shift the characteristic stereospecific distribution of fatty acids was conserved, and evidence consistent with a direct desaturation of 18:1-MGDG and 18:2-MGDG to yield 18:3 at the *sn*-1 position of MGDG was obtained.

Further studies of lipid biosynthesis and metabolism in *Aphanizomenon* sp. were carried out through radiolabelling studies with ^{14}C-acetate and ^{3}H-glycerol. After incubation at 28°, at all time periods examined, there was a substantial incorporation of radioactivity into the total lipid fraction, FAME and each glycerolipid class. The incorporation of each substrate into the *Aphanizomenon* lipid classes was near maximal after a 3h incubation period, whilst after 20h incubation, the proportion of radioactivity decreased. In the glycerolipid fractions the PG fraction was the most highly labelled glycerolipid at each incubation period from either ^{14}C-acetic acid or ^{3}H-glycerol. Amongst the glycolipids MGDG was the most heavily labelled. The *Aphanizomenon* glycerolipid labelling pattern thus was in contrast to that of *A. cylindrica* in which, using^{14}C-acetate as a substrate, whilst PG was substantially labelled, MGDG was the most heavily labelled glycerolipid (Nichols,1969 ; Abreu-Grobois, 1977). The small levels (0.90-0.92 %) of radioactivity incorporated from ^{3}H-glycerol into the FAME fraction at each time period indicated that glycerol is metabolized and converted to fatty acid precursors such as acetyl CoA in *Aphanizomenon* sp.

The level of incorporation of radiolabel in the total lipid, FAME and glycerolipid classes following a temperature transition from 28° to 15° indicated that, in experiments using ^{14}C-acetate alone there was no new synthesis of glycerolipid from the radiolabelled substrate during the first 24h following the temperature shift. In MGDG molecular species during the temperature adaptation, the increase in the radioactivity of species containing 18:3 and the decrease of the radioactivity of molecular species containing 18:1 indicated that the direct desaturation of 18:1 to 18:3 makes a significant contribution during adaptation following a downward temperature shift (Table 6.10). Fig 7.1 shows a proposed pathway for formation

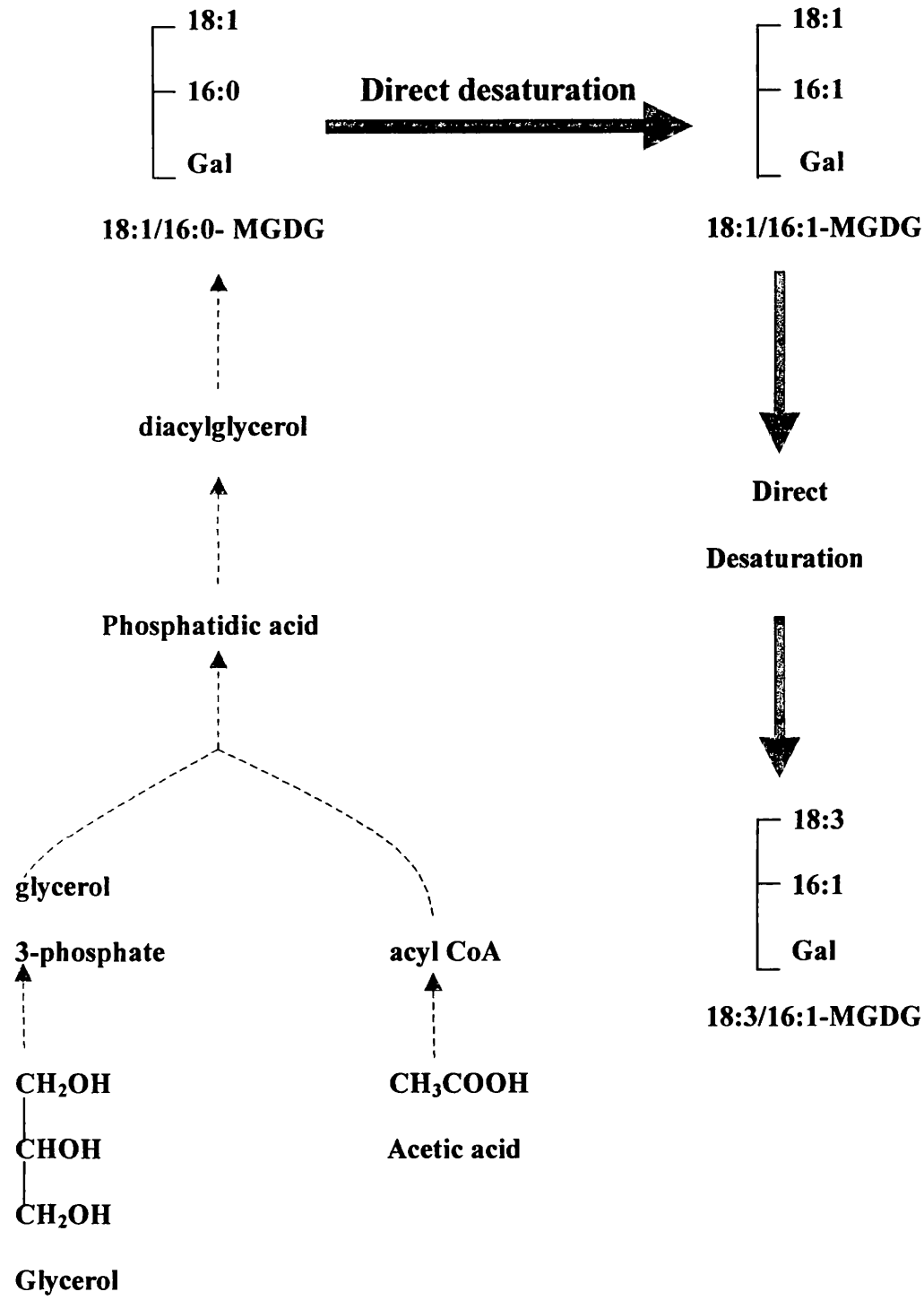


Fig. 7.1 Formation of polyunsaturated MGDG-molecular species during temperature adaptation in *Aphanizomenon* sp

de novo biosynthetic pathway

direct desaturation

of polyunsaturated MGDG- molecular species by direct desaturation during temperature adaptation in *Aphanizomenon* sp.

The mechanism of the temperature shift induced desaturation of the MGDG fraction was further investigated by a ^{3}H:^{14}C double labelling approach. At the end of the pre-labelling period the excess of ^{3}H-glycerol and ^{14}C-acetate substrate was removed by washing the resuspended cells, and 10mM unlabelled sodium acetate was added to dilute out any remaining ^{14}C-radiolabelled substrate (see Chapter 2.13.3); the *Aphanizomenon* cells were then incubated at 15° for the appropriate time period. The incorporation of ^{14}C-acetate into *Aphanizomenon* total lipid was increased by 50% after the first 12h following the downward temperature shift, whilst the radioactivity remained relatively constant after further 12h incubation at 15° (Table 6.12). The increase in the first 12h incubation period indicated that under the experimental conditions there was *de novo* lipid biosynthesis from ^{14}C-acetate and ^{3}H-glycerol which continued during the first 12h following downward temperature shift. The *de novo* synthesis of glycerolipids during the first 12h of the temperature transition in the dual labelling study was in contrast to that using ^{14}C-acetate alone (Table 6.9). It appeared that in the dual labelling experiment, washing of the cells and addition of unlabelled sodium acetate was incapable of preventing biosynthesis of new glycerolipid species from the labelled precursors during the first 12h of transition. It may be possible in future studies to block *de novo* biosynthesis more effectively by use of (i) different pre-incubation conditions (ii) different concentrations of 'cold' substrate (iii) *de novo* lipid biosynthesis inhibitors e.g. cerulenin, which inhibit *de novo* fatty acid synthesis (Omura, 1976). It may also be that the pool of phosphatidic acid formed during the pre-labelling period in the presence of ^{3}H-glycerol was not depleted until 12h after the downward temperature

shift. However, during the second 12h period after temperature shift the level of radioactivity in the MGDG fraction did not increase (Table 6.13) and an increase in radioactivity in MGDG species containing 18:3/16:0 and 18:3/16:1-MGDG between 12h-24h of the transition period and a decrease in the radioactivity in MGDG species containing saturated or monoenoic fatty acid (18:1/16:1-MGDG) was observed (Table 6.14). Furthermore, the similarity of the ^{3}H:^{14}C dpm ratio of MGDG species containing 18:3, in particular 18:3/16:1-MGDG (Table 6.14) isolated 24h after temperature transition to that of 18:1/16:0 formed after 12h at the lower temperature supports the view that direct desaturation of C-16 and C-18 fatty acids occurs during temperature adaptation and provides evidence for the formation of highly unsaturated MGDG molecular species directly from more saturated molecular species in *Aphanizomenon* sp.

Appendix

Appendix 1

Aphanizomenon sp. culture medium (modified ASM 1 freshwater)

NaCl	117.0mg	
$MgSO_4$	36.4mg	
$CaCl\ 2H_2O$	29.1mg	
K_2HPO_4	17.4mg	
Na_2HPO_4	14.2mg	
$NaHCO_3$	66.6mg	
Solution A (fromstock)	10 ml / L	
$CuCl_2$	10µL / L	(13.6mg / L Stock solution)
$CoCl_2$	100µL / L	(19.2mg / L Stock solution)
MoO_3	100µL / L	(14.0mg / L Stock solution)

Solution A stock

H_3BO_3	247.4mg / L
$FeCl_2.6H_2O$	91.0mg / L
$MnCl_2.4H_2O$	110.0mg / L
$ZnCl_2$	44.0mg / L
Na_2EDTA	737.5mg / L

The above components were made up to 1L with double distilled water,
PH checked before autoclaving, should be in the range 7.6-8.0
The pH was adjusted with HCl to 7.6 if necessary.

a) ^{3}H – Counting programme

USER PROGRAM REVIEW			
User: 1	I D: HGAS DPM		
Count time: 5.00			SL DPM
Repeats:	1	H#	Liquid
Replicates:	1		
Cycle Reps:	5		
Low Reject:	0		
Isotope: ^{3}H	NONE		NONE
2 sig: 0.00			
Bkgsub: 0.00			
Factor:1.00000			
Units:	DPM		DPM
Printer: Std		RS-232:	Off

ACTIVE KEYS				
MainC	HelpC	Select		Reset
PrevC	Print	Cancel		

REVIEW/EDIT
Id: HGAS DPM
Comments: SINGLE LABEL DPM ^{3}H
Counting Time: 5.00 min
Isotope 1: ^{3}H
Isotope 2: NONE
Edit Other Parameters

Enter program identification:

b) ^{14}C - Counting programme

USER PROGRAM REVIEW			
User: 6	I D: HGAS ^{14}C DPM		
Count time: 5.00			SL DPM
Repeats:	1	H#	Liquid
Replicates:	1		
Cycle Reps:	1		
Low Reject:	0		
Isotope: ^{14}C	NONE		NONE
2 sig: 0.00			
Bkgsub: 0.00			
Factor:1.00000			
Units:	DPM		DPM
Printer: Std		RS-232:	Off

ACTIVE KEYS				
MainC	HelpC	Select		Reset
PrevC	Print	Cancel		

REVIEW/EDIT
Id: HGAS ^{14}C DPM
Comments: HANA ^{14}C DPM PROGRAM
Counting Time: 5.00 min
Isotope 1: ^{14}C
Isotope 2: NONE
Edit Other Parameters

Enter program identification:

c) $^{3}H+^{14}C$ Dual label counting programme.

USER PROGRAM REVIEW			
User: 8	I D: HGAS $^{3}H+^{14}C$		
Count time: 5.00			CPM
Repeats:	1	H#	Liquid
Replicates:	1		
Cycle Reps:	1		
Low Reject:	0		
Isotope:	^{3}H	^{14}C	NONE
2 sig:	0.00	0.00	
Bkgsub:	0.00	0.00	
Factor:	1.00000	1.00000	
Units:	DPM	CPM	
Printer:	Std	RS-232: Off	

ACTIVE KEYS				
MainC	HelpC	Select		Reset
PrevC	Print	Cancel		

REVIEW/EDIT
Id: HGAS $^{3}H+^{14}C$
Comments:
Counting Time: 5.00 min
Isotope 1: ^{3}H
Isotope 2: ^{14}C
Isotope 3: NONE
Edit Other Parameters

Enter program identification:

Appendix 2

Table 3.1

Concentration of chlorophyll a from culture grown at 28° for 25 days

Age of culture (h)	Chlorophyll conc. mg/100 ml culture
0	0.006
24	0.005
48	0.011
72	0.011
96	0.017
120	0.012
144	0.012
168	0.006
192	0.018
216	0.015
240	0.047
264	0.042
288	0.031
312	0.036
336	0.026
360	0.026
384	0.027
408	0.037
432	0.063
456	0.077
480	0.079
504	0.113
528	0.076
552	0.095
600	0.096

Table 3.2

Concentration of chlorophyll a mg/100ml from culture grown at 28° for 14 days.

Age of culture (h)	Chlorophyll conc. mg/100 ml culture
0	0.002
24	0.002
48	0.003
72	0.005
96	0.006
120	0.008
144	0.037
168	0.037
192	0.040
216	0.044
240	0.046
264	0.048
288	0.096
312	0.099
336	0.094

Table 3.3

Concentration of carotenoid from culture grown at 28° for 25 days.

Age of culture (h)	Conc.of Carotenoid (µg / 100ml)
0	2.20
24	3.20
48	3.50
72	5.68
96	9.68
120	10.80
144	8.90
168	4.48
192	8.92
216	20.80
240	22.64
264	22.32
288	19.04
312	14.16
336	15.44
360	13.04
384	14.50
408	19.92
432	29.36
456	41.60
480	44.00
504	80.20
528	48.00
552	56.60
576	54.40

Table 3.4

Concentration of carotenoid μg /100ml from culture grown at 28° for 14 days.

Age of culture (h)	Carotenoid conc.
0	2.00
24	2.40
48	3.44
72	3.44
96	4.16
120	12.31
144	18.32
168	18.00
192	19.28
216	24.08
240	24.50
264	25.52
288	26.10
312	48.00
336	48.64

Table 3.5

Dry weight from culture grown at 28° for 25 days

Age of culture (h)	Dry wt. (mg) / 100ml culture
0	30
24	30
48	30
72	30
96	30
120	30
144	32
168	32
192	36
216	35
240	37
264	44
288	46
312	46
336	49
360	41
384	37
408	36
432	40
456	40
480	40
504	49
528	49
552	45
600	45

Table 3.6

Dry weight from culture grown at 28° for 14 days

Age of culture (h)	Dry wt. (mg) / 100ml culture
0	26
24	26
48	26
72	26
96	26
120	26
144	29
168	29
192	29
216	33
240	37
264	42
288	44
312	44
336	45

Table 3.7

Concentration of chlorophyll a from culture grown at 15° for 25 days.

Age of culture (h)	Chlorophyll conc. mg/100 ml culture
0	0.008
24	0.008
48	0.007
72	0.006
96	0.008
120	0.012
144	0.011
168	0.040
192	0.027
216	0.034
240	0.022
264	0.057
288	0.054
312	0.035
336	0.033
360	0.050
384	0.056
408	0.058
432	0.072
456	0.060
480	0.096
504	0.112
528	0.116
552	0.138
600	0.096

Table 3.8

Concentration of β- carotene from culture grown at 15 for 25 days

Age of culture(h)	Conc. of β -carotene μg / 100ml
0	16.4
24	7.04
48	6.24
72	4.88
120	10.48
144	6.08
168	16.96
192	13.44
216	17.76
240	12.72
264	17.28
288	27.6
312	17.76
336	21.28
360	29.28
384	29.36
408	17.52
432	12.16
456	34.16
480	28.48
504	30.4
528	40.32
552	43.28

Table 3.9

Dry weight from culture grown at 15° for 25 days

Age of culture (h)	Dry wt. mg / 100ml culture
0	24
24	24
48	26
72	26
96	27
120	28
144	28
168	30
192	32
216	33
240	33
264	32
288	34
312	37
336	38
360	42
384	41
408	42
432	41
456	42
480	44
504	44
528	44
552	43
600	44

Table 4.1 Fatty acid relative % c[redacted]*anizomenon sp.* of total lipid, MGDG, DGDG, PG and SL following a temperature shift from 28° to 15°

Total lipid fatty acid relative % composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0h	37.6	14.6	2.3	3.2	2.3	12.4	7.3	22.3
12h	35.6	16.2	1.6	3.7	2.2	10.3	5.6	24.9
24h	33.7	19.3	1.8	3.9	1.6	11.8	5.2	22.8
36h	33.5	16.3	1.7	5.1	1.8	7.9	4.8	28.9
48h	30.6	17.0	2.0	6.0	1.6	8.3	4.5	30.1

MGDG fatty acid relative % composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0h	31.3	23.6	2.4	4.8	1.6	4.1	4.7	27.6
12h	26.3	20.6	2.3	5.9	0.7	3.3	5.3	35.3
24h	21.6	24.7	3.3	8.7	1.0	3.1	4.3	33.3
36h	19.3	22.0	2.7	7.9	0.7	3.5	3.9	40.0
48h	28.3	18.1	2.8	8.5	0.8	2.9	1.7	37.0

DGDG fatty acid relative % composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0h	30.7	16.4	1.2	4.4	1.5	10.0	6.5	29.4
12h	27.9	15.4	1.2	4.9	1.0	8.2	7.3	34.2
24h	34.9	14.9	2.3	4.4	1.1	6.9	6.1	29.4
36h	32.1	8.3	1.7	4.8	0.7	8.3	6.9	37.1
48h	39.1	11.5	1.6	4.0	1.1	8.0	5.7	29.0

PG relative % composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0h	35.1	10.9	nd	nd	5.8	12.3	7.3	28.7
12h	26.0	12.6	nd	nd	5.3	33.1	5.9	17.2
24h	26.4	16.7	nd	nd	5.3	33.4	4.7	13.6
36h	29.8	9.7	nd	nd	6.8	43.6	8.6	14.1
48h	26.9	16.9	nd	nd	4.6	30.9	3.8	17.9

SL relative % composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0h	50.2	8.3	nd	nd	3.8	17.9	6.0	11.9
12h	44.7	11.0	nd	nd	5.1	14.7	7.4	17.1
24h	49.3	14.4	nd	nd	2.5	13.3	5.7	14.9
36h	46.9	16.5	nd	5.4	2.5	5.6	5.3	17.9
48h	47.7	13.0	nd	3.2	2.7	11.2	4.4	17.2

Table 4.2 Fatty acid relative % comopsition of *Aphanizomenon sp* of total lipid, MGDG, DGDG, PG and SL following a temperature shift from 28° to 15°

Total lipid fatty acid relative % composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0h	37.6	12.5	2.9	5.0	4.0	10.5	5.7	21.7
12h	35.7	13.9	2.9	6.0	3.6	8.9	5.4	23.7
24h	32.2	15.0	1.9	5.8	2.2	10.9	4.5	27.6
36h	33.7	14.9	1.9	7.0	2.0	7.8	4.3	28.5
48h	31.8	13.5	2.1	8.3	1.7	6.9	3.7	32.2

MGDG fatty acid relative % composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0h	29.2	18.0	5.0	9.6	4.2	5.6	5.7	22.8
12h	28.1	17.3	6.5	9.3	5.4	6.1	5.5	21.9
24h	24.9	14.4	2.5	11.5	3.3	3.1	3.6	36.7
36h	27.0	16.0	2.4	9.4	1.2	3.2	4.2	36.3
48h	24.6	13.8	2.2	12.1	1.3	2.6	3.0	40.6

DGDG fatty acid relative % composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0h	36.0	7.8	1.6	7.9	5.6	10.9	4.5	25.9
12h	35.0	9.9	2.0	9.1	4.0	7.2	7.7	26.5
24h	36.0	10.7	1.9	6.2	3.2	6.6	6.3	29.2
36h	35.8	10.2	2.5	4.1	2.0	7.2	6.7	31.6
48h	38.4	10.9	2.6	4.2	2.4	3.9	6.2	31.3

PG relative % composition (28 - 15)

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0h	35.8	11.4	nd	nd	2.2	7.8	4.7	26.4
12h	26.5	9.1	nd	nd	9.0	19.0	4.0	18.0
24h	25.2	15.8	nd	nd	7.6	20.1	4.1	16.9
36h	35.1	10.8	nd	nd	10.8	20.8	nd	16.2
48h	37.2	10.6	nd	nd	5.9	15.9	nd	21.9

SL relative % composition (28 - 15)

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0h	57.0	4.0	nd	nd	9.0	14.0	8.0	8.0
12h	57.5	8.9	nd	nd	7.7	9.7	8.8	7.4
24h	66.5	8.8	nd	nd	7.2	6.8	4.4	6.3
36h	58.3	6.1	nd	5.2	5.7	6.0	5.7	13.0
48h	50.3	5.8	nd	4.9	6.2	7.5	5.8	19.7

Table 4.4 Fatty acid relative % composition of *Aphanizomenon* sp of total lipid, MGDG, DGDG, PG and SL following a temperature shift from 15° to 28°

Total lipid fatty acid relative % composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0	28.8	7.9	1.0	14.5	1.5	4.2	2.3	39.8
12	26.1	8.6	0.9	16.1	1.7	3.9	1.7	41.0
24	29.1	12.7	1.5	12.1	1.1	6.0	1.5	36.1
36	27.6	13.5	nd	12.0	1.5	7.8	1.8	35.8
48	26.1	15.5	1.0	10.9	1.7	8.8	3.0	33.0

MGDG fatty acid relative % composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0	23.2	8.5	1.1	17.3	1.2	1.9	2.0	44.9
12	23.8	8.9	1.0	18.1	1.2	0.8	1.1	45.0
24	25.4	12.4	0.7	11.9	1.5	3.2	1.6	43.3
36	21.4	12.3	nd	15.0	1.3	2.7	3.6	43.8
48	24.5	16.3	nd	12.5	1.0	2.7	3.1	39.9

DGDG fatty acid relative % composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0	31.0	9.9	1.3	13.1	0.8	2.3	2.6	39.0
12	29.9	6.5	1.3	13.8	1.4	2.4	2.5	42.2
24	27.5	11.8	1.2	10.1	1.9	9.7	2.6	35.2
36	23.0	5.1	1.5	15.2	2.1	7.2	3.3	42.7
48	26.9	12.2	1.5	13.8	1.1	2.3	2.5	39.8

PG fatty acid relative % composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0	39.7	9.3	3.1	8.8	7.7	13.7	nd	17.8
12	29.0	26.6	6.2	4.7	3.6	15.7	1.7	12.5
24	31.6	20.0	nd	nd	5.3	29.4	ng	13.7
36	38.1	20.0	nd	nd	4.2	20.3	3.0	14.7
48	27.1	19.3	nd	4.3	5.7	28.4	2.7	12.6

SL fatty acid relative % composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0	47.3	2.7	4.4	4.6	3.0	4.2	2.9	30.3
12	63.6	5.1	4.3	3.7	6.1	7.1	1.9	8.1
24	44.2	4.3	nd	5.6	10.1	17.3	nd	20.3
36	47.0	6.3	nd	3.5	10.1	14.2	nd	18.9
48	51.3	8.2	nd	3.7	4.3	8.3	4.2	20.0

Table 4.5 Fatty acid relative % composition of *Aphanizomenon* sp of total lipid, MGDG DGDG, PG and SL following a temperature shift from 15° to 28°

Total lipid fatty acid relative% composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0	28.2	7.7	0.8	9.0	3.9	17.0	5.8	27.7
12	30.5	9.8	nd	8.9	3.0	16.0	4.4	27.5
24	31.3	9.8	nd	6.0	4.1	20.0	6.6	22.3
36	31.8	11.1	nd	6.2	2.5	17.2	5.5	25.7
48	32.8	12.8	dn	4.8	3.8	20.2	6.9	18.8

MGDG fatty acid relative% composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0	27.4	1096.0	nd	16.7	2.1	10.6	2.1	32.2
12	25.1	11.3	nd	11.0	4.7	10.6	4.7	32.6
24	28.4	9.2	1.8	11.1	1.9	8.4	3.8	35.5
36	26.8	9.3	1.2	10.7	2.3	11.4	5.0	33.5
48	25.2	15.1	nd	11.4	2.4	13.4	4.6	27.9

DGDG fatty acid relative% composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0	29.9	6.6	5.6	8.2	2.1	10.6	3.2	33.8
12	28.1	10.0	nd	9.2	4.0	13.9	3.7	31.2
24	25.0	8.0	nd	12.1	1.9	9.8	3.5	39.6
36	29.4	8.3	nd	8.8	2.8	13.8	4.0	33.9
48	26.5	10.1	nd	12.3	3.5	12.3	5.8	31.6

PG fatty acid relative% composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0	38.2	11.9	nd	9.1	5.3	15.4	4.2	16.0
12	30.2	22.4	3.0	5.3	4.5	18.8	3.0	13.0
24	38.9	14.3	1.6	9.4	2.9	17.1	2.3	13.5
36	30.6	18.6	1.1	4.1	2.9	14.4	4.1	23.2
48	22.5	21.3	1.7	5.6	3.9	27.8	2.3	15.1

SL fatty acid relative% composition

Age of culture (h)	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3
0	45.7	1.6	nd	10.3	6.3	6.3	13.7	19.3
12	49.8	8.7	nd	10.2	7.6	8.9	20.6	18.6
24	34.5	16.5	nd	12.0	6.2	6.2	18.7	12.3
36	48.7	8.7	nd	9.9	3.3	3.3	10.5	15.6
48	46.0	7.1	nd	4.1	4.9	4.9	13.3	18.3

Appendix 4

Table 5.1. Stereospecific analysis of fatty acid distribution for MGDG from *Aphanizomenon* sp. cultured at 28°

	Fatty acid relative % composition			
Fatty acid	Total lipid	MGDG	MGDG sn-2	MGDG sn-1*
C16:0	37.0	28.4	68.8	-12.0
C16:1	15.4	18.5	18.1	19.0
C16:2	4.5	2.8	3.0	2.6
C16:3	2.6	3.2	5.1	1.3
C18:0	1.3	2.0	0.5	3.4
C18:1	13.1	13.2	0.4	26.0
C18:2	4.9	7.5	0.5	14.4
C18:3	21.2	24.4	3.6	45.2

* calculated by difference (see Chapter 2)

Table 5.2 Stereospecific analysis of fatty acid distribution for DGDG from *Aphanizomenon* sp. cultured at 28°

	Fatty acid relative % composition			
Fatty acid	Total lipid	DGDG	DGDG sn-2	DGDG sn-1*
C16:0	37.0	30.9	86.5	-24.0
C16:1	15.4	14.6	nd	29.2
C16:2	4.5	2.1	2.6	1.6
C16:3	2.6	3.3	2.9	3.6
C18:0	1.3	2.1	2.1	2.1
C18:1	13.1	13.5	2.9	24.2
C18:2	4.9	7.1	nd	14.2
C18:3	21.2	26.4	2.9	49.9

* calculated by difference (see Chapter 2)

Table 5.3 Stereospecific analysis of fatty acid distribution for PG from *Aphanizomenon* sp. cultured at 28°

	Fatty acid relative % composition			
Fatty acid	Total lipid	PG	PG sn-2	PG sn-1*
C16:0	37.0	37.7	48.2	27.1
C16:1	15.4	7.4	24.1	-9.3
C16:2	4.5	nd	nd	nd
C16:3	2.6	nd	nd	nd
C18:0	1.3	7.9	27.7	-11.8
C18:1	13.1	28.6	nd	57.1
C18:2	4.9	5.3	nd	10.7
C18:3	21.2	13.1	nd	26.2

*** calculated by difference (see Chapter 2)**

Table 5.4 Stereospecific analysis of fatty acid distribution for SL from *Aphanizomenon* sp. cultured at 28°

	Fatty acid relative % composition			
Fatty acid	Total lipid	SL	SL sn-2	SL sn-1*
C16:0	37.0	62.8	94.5	31.0
C16:1	15.4	nd	nd	nd
C16:2	4.5	nd	nd	nd
C16:3	2.6	nd	nd	nd
C18:0	1.3	3.0	5.5	0.6
C18:1	13.1	12.8	nd	25.5
C18:2	4.9	6.3	nd	12.7
C18:3	21.2	15.1	nd	30.2

*** calculated by difference (see Chapter 2)**

Table 5.5 Stereospecific analysis of fatty acid distribution for MGDG from *Aphanizomenon* sp. cultured at 15°.

	Fatty acid relative % composition			
Fatty acid	Total lipid	MGDG	MGDG sn-2	MGDG sn-1*
C16:0	26.0	20.5	55.3	-14.2
C16:1	6.7	7.1	nd	14.3
C16:2	1.2	2.0	4.0	0.0
C16:3	15.0	18.7	35.9	1.5
C18:0	0.7	0.3	nd	0.5
C18:1	2.7	1.3	0.4	2.2
C18:2	2.8	2.0	nd	4.0
C18:3	44.9	48.1	4.5	91.7

*calculated by difference (see Chapter 2.12.3)

Table 5.6 Stereospecific analysis of fatty acid distribution for DGDG from *Aphanizomenon* sp. cultured at 15°

	Fatty acid relative % composition			
Fatty acid	Total lipid	DGDG	DGDG sn-2	DGDG sn-1*
C16:0	26.0	29.1	72.6	-14.3
C16:1	6.7	9.3	nd	18.7
C16:2	1.2	1.4	3.2	-0.3
C16:3	15.0	11.3	19.9	2.6
C18:0	0.7	0.5	nd	1.0
C18:1	2.7	2.7	nd	5.5
C18:2	2.8	2.9	nd	5.8
C18:3	44.9	42.7	4.4	81.0

*calculated by difference (see Chapter 2.12.3)

Table 5.7 Stereospecific analysis of fatty acid distribution from PG from *Aphanizomenon* sp. cultured at 15°

	Fatty acid relative % composition			
Fatty acid	Total lipid	PG	PG sn-2	PG sn-1*
C16:0	26.0	26.9	51.3	2.5
C16:1	6.7	15.1	nd	30.1
C16:2	1.2	nd	nd	nd
C16:3	15.0	7.0	37.4	-23.4
C18:0	0.7	6.0	nd	12.0
C18:1	2.7	27.5	nd	55.1
C18:2	2.8	4.8	nd	9.7
C18:3	44.9	12.7	11.3	14.1

*calculated by difference (see Chapter 2.12.3)

Table 5.8 Stereospecific analysis of fatty acid distribution for SL from *Aphanizomenon* sp. cultured at 15°.

	Fatty acid relative % composition			
Fatty acid	Total lipid	SL	SL sn-2	SL sn-1*
C16:0	26.0	60.5	100.0	20.0
C16:1	6.7	nd	nd	nd
C16:2	1.2	nd	nd	nd
C16:3	15.0	nd	nd	nd
C18:0	0.7	1.1	nd	2.3
C18:1	2.7	4.2	nd	8.5
C18:2	2.8	3.4	nd	6.9
C18:3	44.9	30.7	nd	61.5

*calculated by difference (see Chapter 2.12.3)

Appendix 5

Table 6.2a Incorporation of radioactivity from sodium [1-^{14}C] - acetate into the major glycerolipid classes in *Aphanizomenon* sp.

Incubation	Radioactivity (dpm)*			
Time / h	MGDG	DGDG	PG	SL
1	75743	57230	102223	74443
3	72913	50700	101260	67413
6	107410	78420	117910	74980
20	92536	41493	146473	77000

*values are average d.p.m values of duplicate 30µl aliquots: from a total lipid sample of 0.1ml total volume

Table 6.3 Incorporation of radioactivity from sodium [1-^{14}C] - acetate into the major glycrolipid classes in *Aphanizomenon* sp.

Incubation	Radioactivity (dpm)*			
Time / h	MGDG	DGDG	PG	SL
1	28473	27180	33230	20930
3	39783	26203	47870	27176
6	44113	8413	55936	28710
20	57070	22113	75326	41800

*values are average d.p.m values of duplicate 30µl aliquots: from a total lipid sample of 0.1ml total volume.

Table 6.5 Incorporation of radioactivity from [1-^{3}H]- glycerol into the major glycerolipid classes in *Aphanizomenon* sp.

Incubation	Radioactivity (dpm)*			
Time / h	MGDG	DGDG	PG	SL
1	131793	104803	885630	98047
3	237303	134937	814693	111197
6	294663	38483	712480	116863
20	151503	62673	474383	77877

*values are averages of duplicate aliquots.

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